Molecular mechanisms of myricetin bulk and nano forms mediating genoprotective and genotoxic effects in lymphocytes from pre-cancerous and myeloma patients

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Submitted for the Degree of Doctor of Philosophy

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2018

Abstract

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Key words: myricetin; bulk and nano forms; genotoxic; genoprotective; human lymphocytes; pre-cancerous blood disorders; myeloma patients; healthy individuals; PhIP and H_2O_2

Cancer is one of the leading causes of death across the globe which needs appropriate and cost-effective treatment. Several recent studies have suggested that dietary intake of various flavonoids such as myricetin have a protective effect against different types of cancers and cardiovascular diseases. The present study was conducted to investigate the genoprotective and genotoxic effects of myricetin nano and bulk forms on the lymphocytes from pre-cancerous and multiple myeloma cancer patients compared to those from healthy individuals. Also, to investigate the protective potential of myricetin bulk and nano against the oxidative stress produced *in vitro* by 2- amino-1-methyl-6 phenylimidazo [4, 5-b] pyridine and reactive oxygen species- induced DNA damage using the Comet assay, micronucleus assay, cellular reactive oxygen species and glutathione detection assay, Western blotting, real-time polymerase chain reaction and immunofluorescence. Lymphocytes from the patient groups showed significantly higher levels of basal DNA damage compared to the lymphocytes from healthy individuals which was observed throughout the *in vitro* treatment.

Myricetin in both forms has not induced any significant DNA damage in all of the investigative groups at selective lower concentrations; in fact, the results demonstrate a reduction in DNA damage upon treating with myricetin nano in lymphocytes from pre-cancerous patients demonstrated by significant reduction in micronuclei formation in mononucleated cells. DNA repair capacity of myricetin bulk and nano was determined by co-treating the drugs with hydrogen peroxide. Myricetin significantly reduced the oxidative stress related damage caused by hydrogen peroxide, where myricetin nano seemed to be more effective employing the Comet assay. In the presence of myricetin bulk and nano, the damaging effects of 2- amino-1-methyl-6 phenylimidazo [4,5-b] pyridine were considerably decreased, where myricetin nano was more effective. This could be because nanoparticles have a larger surface area which could improve their reactivity and also the reduction in size of the particles could improve the anti-cancer properties of this compound.

Myricetin has shown genoprotective and anti-oxidant effects by demonstrating the potential to reduce DNA damage caused by over-production of reactive oxygen species and oxidative stress. It has also shown anti-cancer potential in the lymphocytes from multiple myeloma patients by regulating the apoptosis related proteins, dependent on oxidative stress. Therefore, this study suggests that myricetin supplementation in our regular diet with enhanced bioavailability could have potential health beneficial effects and possibly protect against various diseases including cancer.

Dedication

This work is dedicated to my ever-faithful ALLAH (SWT) for keeping me on right path, strong and focused.

To the memory of my late father, Major Ali Bahadur, only because of him, I could avail this opportunity to pursue a PhD in UK.

To my super-woman mother, Mrs Zeenat Begum, for believing in me since I was a child, for bringing me up like a lioness, for all her love, prayers and emotional support. I could not have had imagined coming this far without her.

Most importantly, to my loving husband, Mr Nasir Qayyum, nothing was possible without his backing, love, emotional and financial support. He has served like an angel all these years.

To my siblings, Robina Akhtar, for her inspiration, love and physical support throughout my studies and Shabaz Ali, for his love.

Last but not least, to my little daughter princess, Abir, for enduring my absences and compromises we had to make all this time.

Acknowledgements

I would like to express my gratitude to my supervisors for their great mentorship. To Professor Diana Anderson (Established Chair of Biomedical Sciences, University of Bradford), thanks to her for the continuous support and motivation. Her immense knowledge, abilities and advice is priceless. To Dr Rajendran C Gopalan, for all the support he provided with different aspects of this research work. Without his guidance and knowledge, my write-up would have been less organised. I am grateful to Dr Mojgan Najafzadeh, who has kindly arranged and provided me with the patient blood samples, taken by the staff from Bradford Royal Infirmary (BRI). To Dr Mohammed Isreb, for the preparation of nanoparticles and Stuart Fox, for the training and assistance he provided with the transmission electron micrography.

My sincere thanks also go to Emtiaz Aziz for his technical support on a daily basis. He made my lab life enjoyable. I thank my colleagues (Khaled, Mahmood, Emmanuel, Osama, Azeza and Omera) for the memorable time we have had over the years. My special thanks to Dr Anthony Anene for being always there to provide me with his unconditional support and exchanging wonderful ideas. Last but not least; I would like to thank G-floor technical staff for their help with providing equipment. To, all those volunteers who supported me by donating their blood and made this research possible.

Declaration

I solemnly declare that this work is my own work and where the work of others is used, has been correctly acknowledged. The content of my thesis has not been submitted for the award of PhD elsewhere.

Table of content

Contents

List of figures

List of tables

Abbreviations

8-OHdG: 8 hydroxy deoxyguanosine

oxoG: 7, 8-dihydro-8-oxoguanine

A: Adenine

AIA: Aminoimidazoazaarenes

ALL: Acute lymphoblastic leukemia

ALS: Alkali labile site

AML: Acute myeloid leukemia

ANOVA: Analysis of variance

APS: Ammonium persulphate

apoB_{100} protein: Apolipoprotein B 100

ATP: Adenosine 5' triphosphate

ATM: Ataxia telangiectasia mutated

ATR: ATM-Rad3-related

ATRIP: ATR-interacting protein

BiNC: Binucleated cells

BSA: Bovine serum albumin

BER: Base Excision Repair

BRI: Bradford Royal Infirmary

C: Cytosine

CAT: Catalase

cDNA: Chromosomal deoxyribonucleic acid

CB: Carbon black

CBMN: Cytokinesis-Block Micronucleus assay

CCD: Charge coupled device

Cdc25A: Cell division cycle 25A

Cdc25C: Cell division cycle 25C

COX: Cyclo-oxygenase

CdKs: Cyclin dependent kinases

CDKIs: Cyclin dependent kinase inhibitors

CYP24: Catalyses 1a, 25-$[OH]_2$-vitamin D

CYP27B1: Catalyses 25-OH-vitamin D

CYP1A2: Cytochrome P-4501A2

CHD: Coronary heart diseases

CLL: Chronic lymphoblastic leukemia

CML: Chronic myeloid leukemia

CNS: Central nervous system

CO_2: Carbon dioxide

CYT-B: Cytochalasin B

$C_6C_3C_6$: Diphenylpropane

Di MeIQx: 2-amino-3,4,8-trimethylimidazo[4,5-*f*] quinoxaline

DDR: DNA damage response

DD-H_2O: Double distilled water

dH_2O: Distilled water

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

DNA-PK: DNA-dependent protein kinase

DSB: Double strand break

dsDNA: Double stranded deoxyribonucleic acid

DTT: Dithiothreitol

DPX: Distyrene, plasticizer, and xylene containing mountant

EB: Ethidium bromide

EDTA: Ethylene diamine tetra acetic acid

ECL: Enhanced chemiluminescence

EGF: Epidermal growth factor

EM: Excipient mixture

EpRE

e.g.: For example

etc: etcetera

F: Female

FBS: Foetal bovine serum

G: Guanine

g : gram

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GADD45: Growth Arrest and DNA Damage 45

GAM: GSH assay mixture

G1 and G2: Gap phase 1 and Gap phase 2

GDP: Guanosine 5'-diphosphate

GOF: Gain of function

GTP: Guanosine 5'-triphosphate

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Reduced glutathione -S-transferase enzyme

GSSG: Oxidised glutathione

H: Hydrogen ion

HL: Hodgkin lymphoma

H_2O_2: Hydrogen peroxide

HCAs: Heterocyclic amines

HCl: Hydrochloric acid

HEp-2: Human epithelial cervical carcinoma (HeLa) cells

HL-60: Human promyelocytic leukaemia cell line

Hrs: Hours

HPMC: Hydroxypropyl methylcellulose

HR: Homologous recombinant

HUMN: Human MicronNucleus

hprt: Hypoxanthine-guanine phosphoribosyl transferase

Igs: Immunoglobulins

IQ: 2-amino-3-methylimidazo[4,5-*f*] quinoline

i.e: That is

IF: Immunofluorescence

ICC: Immunocytochemistry

IOM: Institute of medicine

KCl: Potassium chloride

kDa: Kilo-Dalton

LDL: Low density lipoprotein

LMP: Low melting point

M: Male

mA: Milli ampere

M phase: Mitosis

Mdm2: Mouse double minute 2 homolog

MeIQ: 2-amino-3,4-dimethylimidazo[4,5-*f*] quinolone

MeIQx: 2-amino 3,8dimethylimidazo[4,5-*f*] quinoxaline

MGUS: monoclonal gammopathy of undetermined significance

ml: Millilitre

mg: Milligram

mg/ml: Milligram per millilitre

mins: Minutes

MM: Multiple myeloma

MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

MMC: Mitomycin C

MMP: Matrix metalloproteinase

MN: Micronucleus

MNi: Micronuclei

MoNC: Mono-nucleated cells

MultiNC: Multi nucleated cells

MRS: Mismatch repair system

MYR B: Myricetin bulk

MYR N: Myricetin nano

N-OH-PhIP: 2-hydroxyamino-1-methyl-6- phenylimidazo [4,5-*b*] pyridine

NaCl: Sodium chloride

NATs: N-acetyl-transferases

NAD: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphatase

NaOH: Sodium hydroxide

Na_2HPO_4: Disodium hydrogen phosphate

NaH_2PO_4: Sodium di-hydrogen phosphate

NC: Negative control

NCI: National cancer institute

NDI: Nuclear division index

NER: Nucleotide excision repair

NF-kB: Nuclear factor kB

NIH: National Institute of Health

NFDM: Non-fate dry milk

NHEJ: Non-homologous end joining

NHL: Non-Hodgkin lymphoma

NMP: Normal melting point

NPBs: Nucleoplasmic bridges

NPs: Nanoparticles

nm: Nano metre

OTM: Olive tail moment

O_2: Oxygen ion

OH: Hydroxyl ion

ºC: Degree centigrade

OD: Optical density

P/S: Penicillin streptomycin mix

PAGE: Polyacrylamide gel electrophoresis

PAs: Pyrrolizidine alkaloids

PBS: Phosphate buffer saline

PC: Positive control

PC-3: Prostate cancer cell line

PCD: Programmed cell death

PCR: Polymerase chain reaction

PCNA: Proliferating cell nuclear antigen

PDGF: Platelet derived growth factor

PHA: Phytohaemagglutinin

PhIP: 2- amino-1-methyl-6 phenylimidazo [4,5-b] pyridine

pmol/l: Pico moles per litre

PIKKS: Phosphoinositide 3-kinase-related protein kinases

PK: Proteinase K

PVP: Polyvinylpyrrolidone

PVDF: Polyvinylidene fluoride or polyvinylidene difluoride

RBCs: Red blood cells

Rb: Retinoblastomas

ROS: Reactive oxygen species

rpm: Revolutions per minute

RNA: Ribonucleic acid

RT: Room temperature

RPMI: Roswell Park Memorial Institute

RPA: Replication protein A

S phase: Synthesis phase

Secs: Seconds

SLS: Sodium lauryl sulphate

STM: Scanning tunnel microscope

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SE: Standard error

SGCE: Single cell gel electrophoresis

ST: Simultaneously treated

SOD: Superoxide dismutase

SPSS: Statistical package for social sciences

SSBs: Single strand breaks

ssDNA: Single stranded deoxyribonucleic acid

SULTs: Sulfotransferases

T: Thymine

TBHP: Tertiary-butyl hydro peroxide

TEM: Transmission electron microscope

TGF-β: transforming growth factor β

tRNA : Translational ribonucleic acid

TBS-T: Tris buffer solution with tween-20

TEMED: N, N, N',N' tetramethyl ethylenediamine

TGAM: Total GSH assay mixture

TP53: Mutant P53

TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand

UK: United Kingdom

UV: Ultra violet

V: Volts

v/v: Volume per volume

VDR: Vitamin D receptor

WHO: World Health Organization

WHO/IPCS: World Health Organisation International Programme for Chemical Safety

WBCs: White blood cells

w/w: Weight per weight

w/v: Weight per volume

WTP53: Wild-type P53

XRCC4: X-ray-complementing Chinese hamster gene 4

µg: Micro gram

µl: Micro litre

µM: Micro molar

M: Molar

mM: Milli molar

β: Beta

γ-H2AX: Gamm H2AX

^{0}C: Degree centigrade

cm^{3}: Centimetre cube

mm^{2}: Millimetre square

Chapter 1: Introduction

1.1 General Introduction

Cancer is considered to be an important cause of death across the world. It accounted for 8.2 million deaths in 2012 and according to the World Health Organisation (WHO, 2016); the number of cases is due to increase from 14 to 22 million within two decades. Being second leading cause of death globally, cancer is responsible for 1 in 6 deaths (WHO, 2018). Cancer is defined as a rapid transformation of normal cells into malignant tumours as a multistage process, interacting with a person's genetic factors and environmental factors (Doll and Peto, 1981; WHO, 2018). Early detection and treatment of cancer can notably make the difference in prognosis of the disease, thereby reducing the damage and financial burden. Biological markers play a crucial role in early diagnosis of cancer.

Most of the human genome is comprised of deoxyribonucleic acid (DNA) sequences that do not code for proteins. Protein coding sequences only make up 1.5% of the genome. There are two types of nucleotide sequences within a gene: exons (coding) and introns (non-coding) (Alberts et al., 2002). The mutations in exons alone affect the amino acid protein composing sequences. Cancers start from a single somatic cell then a series of genetic changes occur in the cells of the emerging neoplasm which lead to alterations in gene function and phenotype therefore causing tumour development (Ponder, 2001; NCI, 2018).

Various studies have been conducted in the past few decades in order to understand the cell cycle processes and those, involved in transforming a normal cell to a cancerous cell. A manifestation of various important alterations gives rise to most of the cancer cells' genotypes that together dictate cancer

progression. In order for cancer to develop, a cell must acquire several capabilities: independence of external growth signals, insensitivity to anti-growth signals, evade apoptosis, immortality, be able to trigger angiogenesis and become metastatic. However, several types and subtypes of cancer have made it difficult to determine a specific tumour's aetiology and origin.

The focus of our current study is the pre-cancerous state and blood cancer, especially, multiple myeloma (MM). Following is a brief description of various blood cancer types.

1.2 Blood Cancer and its types

Blood cancer is a canopy term used for the cancers that are associated with the blood, lymphatic system and bone marrow. Sadly, a large number of people are affected by blood cancer daily, worldwide. The three main types of blood cancers are leukemia, lymphoma and myeloma.

1.2.1 Leukemia

Leukemia is the cancer of leukocytes where an abnormal number of immature cells is produced which are gathered up in bone marrow forming masses and disrupts the production of other important cell types. These abnormal white blood cells are rapidly produced in large numbers and enter the main blood stream so that these leukaemia cells crowd out the normal blood cell number, leading to serious problems e.g. anaemia.

Depending on the degree of progression, it is divided into two types: acute, develops and progresses quickly needing urgent treatment (Appelbaum, 2006) whereas chronic, develops gradually over months and years. Depending on which cell type is affected, it can be further categorised into lymphocytic or

myelogenous leukaemia. The former is leukaemia of white blood cells and the later affects other cell types including erythrocytes and platelets (Kantarjian and O'Brien, 2011). The standard staging system for leukaemia has not yet been developed but symptoms can be monitored and prevented using certain treatments.

1.2.1.1 Acute myeloid leukemia (AML)

This is a cancer of myeloid cells which occurs in bone marrow. Normally, myeloid cells develop into different cell types i.e. RBCs, platelets and WBCs, whereas in AML growth stops at a very young and developing stage forming immature myeloblasts in an uncontrolled manner. Meanwhile these immature cells multiply quickly stopping normal growth of blood cells. AML is rare in the UK and it is more common in people over 70 years of age.

1.2.1.2 Chronic myeloid leukemia (CML)

This is very rare cancer, of a particular myeloid cell called a granulocyte, causing overproduction of these cells in bone marrow and affects normal growth of other blood cells.

1.2.1.3 Acute lymphoblastic leukemia (ALL)

ALL is a cancer involving lymphocytes from the WBCs. At the immature developmental stage of lymphocyte the cell is said to be a lymphoblast then it develops into a fully functional lymphocyte. In ALL, cells stop their growth at this immature stage and multiply quickly affecting the proper working of lymphocytes in the immune system. Clusters of lymphoblasts clog up in bone marrow

distressing other blood constituents. It is rare in adults but a very common leukemia in children in the UK (Kantarjian and O'Brien, 2011).

1.2.1.4 Chronic lymphoblastic leukemia (CLL)

CLL affects the function of lymphocytes and causes over production of lymphoblasts over a period of time and slowly fills up the bone marrow with these undeveloped lymphocytes reducing the number of other blood cells essential for bodily functions. It may also cause big, swollen lymph nodes. It is common in elderly people.

1.2.2 Lymphoma

Lymphoma is caused by excessive production of lymphocyte affecting our lymphatic system which plays an important role in balancing the immune responses and provides protection against infection and disease. It can develop in the blood, spleen, lymph nodes, bone marrow and other organs. There are two main types of lymphoma:

Hodgkin lymphoma (HL): In this type of lymphoma, cancer originates from lymphocytes and it can develop at any age but most commonly occurs in young adults.

Non Hodgkin lymphoma (NHL): Most lymphomas are NHL. It is the sixth most common cancer in UK, affecting typically people over 70 years of age (https://www.anthonynolan.org/patients-and-families/blood-cancers-and-blood-disorders/what-blood-cancer/lymphoma).

1.2.3 Myeloma

Plasma cells are a type of B-lymphocytes WBCs forming an important part of our blood, found in bone marrow and produce antibodies to help fighting against infection. Plasma cells normally produce antibodies composed of two types of proteins; light chains and heavy chains, two of each assembled in Y shape. Five main types of antibodies or immunoglobulins (Igs) are produced by our plasma cells known as IgG, IgA, IgM, IgD or IgE. In myeloma, clogging up of abnormal plasma cells in bone marrow stops those from producing an important part of our immune system instead producing excessive abnormal antibodies called paraprotein or monoclonal gammopathy or M protein in blood and bones which is not able to fight infection and its presence is an initial and important sign of myeloma. Therefore, MM, commonly called myeloma is cancer of these plasma cells. It is a neoplastic disorder involving plasma cells that is categorized by colonialization and proliferation of malignant plasma cells in the bone marrow (Palumbo and Anderson, 2011). It can occur anywhere in the body where there is bone marrow present including the spine, ribcage and pelvis, which is why it is often called MM.

People over 70 years of normally get myeloma and it does not occur in children. There are two types of myeloma; asymptomatic, showing no symptoms or tissue damage and symptomatic, expressing symptoms and tissue damage. There are no known causes of this neoplasm but it is always preceded by a premalignant state called monoclonal gammopathy of undetermined significance (MGUS) (Landgren et al., 2009; Weiss et al., 2009). MGUS is a condition where small number of plasma cells starts producing paraprotein but does not have myeloma at this stage. People who suffer from MGUS are at higher risk of getting myeloma. It is quite a common disorder in UK. Around 1 in 100 people with MGUS develop myeloma each year. The precise risk of

myeloma development depends on several factors including the concentration and type of monoclonal protein, plasmacytosis of bone marrow, ratio of serum free light chains and percentage of phenotypically clonal plasma cells (Rajkumar et al., 2014). There is no permanent cure for myeloma and it always relapses. However, chemotherapy using combination of drugs, corticosteroids and targeted therapies are normally used to treat the symptoms, slow down the proliferation, increase the survival rate and to improve the quality of life of patient (Rajkumar, 2011; Rajkumar et al., 2014). Likewise, diet, enriched with fruits and vegetables has been shown to alter the risk of developing MGUS as well as advancement to MM (Thordardottir et al., 2016; Thordardottir et al., 2018).

1.3 Biomarkers of cancer

According to the Institute of Medicine (IOM) a biomarker can be described as a property that can justly measure various biological samples and that can be assessed as an exposure marker of regular or pathogenic biological mechanisms or pharmacologic responses to a certain intervention (Corella and Ordovas 2015).

The most commonly used biological samples include blood, plasma, serum, faeces, saliva, nails, urine and different tissue samples. Biomarkers of exposure and disease status can be determined from these samplings. Biomarkers can be used for diagnostic reasons, to determine disease status, to suggest prognosis of disease and to help with the effective treatment of any disease. Biomarkers are also used for designing and formulation of diagnostic classifications (Ceusters and Smith 2015).

1.4 Dietary nutrients and disease prevention

Dietary requirements for prevention of DNA damage and that of deficiency diseases are different. The diet may affect the basic level of DNA damage rates and may have radiation protective effects. Several *in vitro* and *in vivo* studies showed that concentrations of dietary constituents (folate and vitamin B12) (e.g. 150pmol/l vs 300pmol/l respectively) recommended against anaemia and other deficiency diseases, are not sufficient for prevention of DNA damage (Fenech, 2009). It has been proven that these minor differences in dietary concentrations are as proficient at causing DNA damage as high as mutagens or other cancer-causing agents. A series of papers by Michael Fenech promoted this concept that recommendations for dietary concentrations should be made considering DNA damage as a large factor. Because this could be a most important cause of multiple disorders as genome damage is implicated (in cancer, infertility, ageing etc.) (Fenech, 2001).

Research has found calcium and vitamin D are chemo preventative aids against colon cancer in humans, by understanding their mechanism of action in reducing the risks related to disease and establishing treatable risk biomarkers, for colon cancer. Studies based on fossils and human-gatherings etc. has proposed that our modern dietary intake of calcium and vitamin D is very low as compared to then (1500-2000 mg/day) which could increase risk of chronic diseases especially colon cancer. That intake was even greater than the present recommendation which is between 1000-1200mg/day among different gender and age groups. This is certainly due to today's life style, clothes and less ultraviolet light exposure. Vitamin D was primarily considered in maintaining bone and calcium homeostasis but now the vitamin D receptor

(VDR), CYP24 (catalyses 1a, 25-$[OH]_2$-vitamin D) and CYP27B1 (catalyses 25-OH-vitamin D). These are abundantly expressed in the colon and other tissues, where vitamin D has autocrine/paracrine functions. It regulates over 200 genes, highly involved in colorectal tumorigenesis (McCullough et al., 2009).

Similarly flavonoids, semi-essential food components, secure great importance in our modern research by exhibiting incredible health beneficial effects against various chronic diseases. However, estimating their amount in our diet is a first step towards listing their protective effects. Studies have shown their daily intake estimation between 20mg-1g in Western countries (Chun, 2007). These results could vary due to different confounding factors associated with these studies including differences in antioxidant compounds, their strengths, doses and the characteristics of the subjects involved, etc. An epidemiological study showed an inverse correlation between the coronary heart disease in elderly males and the intake of flavonoids (Hertog et al., 1993). The pharmacological and biological attributes of flavonoids are comprehensively studied. In addition to their free radical scavenging property, they are capable of various biological actions including vasodilation, anti-inflammatory, anti-carcinogenic, anti-viral and bacterial, immune-stimulatory as well as inhibiting glutathione reductase (Elliott et al., 1992).

1.5 The flavonoids and their role in human nutrition

Research shows that utilization of a low fat diet with enhanced fruits and vegetables, particularly enriched with phenolic compounds may help to reduce the risk of many life threatening diseases. Phenolic compounds are present in many fruits, vegetables and nuts etc. These compounds have been utilized by humans since their existence on this earth. There are thousands of phenolic

compounds found and are described. These compounds are produced by plants as secondary metabolites important in various responses including development, fertilization and fighting against pathogens. These plant pigments are found in all types of foods of plant origin (Kyselova, 2011). The main dietary sources of polyphenols are fruit and beverages (tea, coffee, wine, fruit juices, beer and chocolate) and to a lesser amount vegetables, cereals and dry legumes (Augustin and Williamson, 2000). Flavonoids are a major group of phenolic compounds which are contributed by edible plants to the human diet but the precise nature of these compounds is not known. They are classified as low-molecular-weight polyphenolic compounds that are ubiquitously present in fruit and vegetables, bearing a diphenylpropane ($C_6C_3C_6$) skeleton, and categorised according to their chemical structure, into flavonols, flavones, flavanones, isoflavones, anthocyanidins and chalcones (Kurzawa, 2012). Each group is different from the other on the basis of the number and arrangement of hydroxyl groups and to the degree and extent to which these groups undergo glycosylation. Those with dihydroxylation in positions 3' and 4' on B ring mostly occur in flavones and flavonols. The ideal glycosylation site on the flavonoids is in position 3' and seldom in position 7'. The most common sugar residue is glucose but others could be galactose, xylose and rhamnose (Rice-evans et al., 1995). Most of the flavonols and flavones demonstrate two key absorption patterns in the visible region/ultraviolet; B ring absorption characterised by Band I in the 320-385nm range and Band II in the 250-285nm range indicating the A ring absorption. Increase in the number of hydroxyl groups increase absorption band position (Mabry et al., 1970). The presence of the hydroxyl group at position 3 (3-OH) on the B ring of flavonols distinguishes them from flavones. This means Band I of flavonols is always at a longer wavelength by 20-30nm

than in the corresponding flavones. Flavanones possess saturated heterocyclic C ring (without a double bond), lacking consequent conjugation between rings A and B as compared to flavonols and flavones, contributing to their lower antioxidant activity (Mabry et al., 1970). Hence the C ring unsaturation is important for electron delocalisation across the molecule and to stable aryloxyl radicals.

Fruit and vegetable intake could help to reduce atherosclerosis, hypertension, thrombosis, inflammatory processes related to cardio-vascular diseases and many other health issues. The protective effects are largely contributed by the antioxidants derived from antioxidant nutrients vitamin C, E and β carotene. Also minor carotenoids and plant phenolic compounds such as flavonoids may also have a significant role by acting as antioxidant or a mediator of other involved mechanisms. Plant phenolics are capable of performing various actions acting as a reducing agent, oxygen scavenger and oxidising antioxidant by donating a hydrogen ion. Their antioxidant activity is conferred by the hydroxyl groups attached ring structures and predicted by their chemical property of having phenolic hydrogens, available as hydrogen donating radical scavengers (Halliwell, 1990). Also by their radical's lower reduction potential as compared to those of superoxide and alkyl peroxyl which means flavonoids could disable these species and prevent the consequences of their harmful reactions (Jovanovic et al., 1992). Research has shown the possible health benefit of a flavonoid enriched diet that it could modulate expression of various genes directly linked to disease risk. Similarly the potential of flavonoids to defend against circulatory disease has been summarized by many substantial studies. Research has also shown the ability of anthocyanins as useful agents in triggering apoptosis in leukaemia cells by inducing oxidative stress (Guarrera

et al., 2007). Various features of these compounds are parallel to those of anticancer drugs comprising attachment and cleavage of DNA and the production of ROS in the presence of transition metals ions (Arif et al., 2015). Studies have shown that the consumption of flavonoids can lower the mortality rate caused by coronary heart disease (CHD) (Kaur et al., 2007).

Due to various reasons including structural differences, lack of analytical methods, variations in particular food contents, it is extremely difficult to estimate the daily intake of polyphenols but most authors refer to data published many years ago (Ku¨hnau, 1976) where a total daily intake of phenols was reported as 1g.

Little is known about the bioavailability, metabolism and absorption of flavonoids and it is likely that various groups have different pharmacokinetic characteristics (Figure 1.1a,b). Biological characteristics of these compounds depend on their bioavailability. The antioxidant capacity of the plasma increases by the indirect evidence of their absorption through the gut barrier. And more direct evidence of their absorption has been obtained by measuring the concentration in plasma and urine after ingestion of polyphenol rich food. Chemical structure is the main determinant of levels of intestinal absorption and the nature of metabolites circulating in the plasma. Studies showed that the amounts of excreted polyphenol in urine vary from one phenolic compound to another. The absence of polyphenols in urine implies that either they have not been absorbed by the gut barrier, digested and discharged in the bile, processed by communal microflora or absorbed by our own tissues (Hollman et al., 1995). A study proved that catechin is absorbed by the human gut and the plasma level of its major urinary metabolite, a sulphate of 3-O methyl catechin, reached a peak

within two hours of administration (Das, 1971). Most flavonoids rarely reach 1μM concentration in our plasma when the amount of polyphenols does not exceed what is normally consumed in our diet as our diet varies. This concentration is usually achieved within 1-2 hours of ingestion except for the ones which are partially degraded by the clonal microflora. After elimination of a half-life period (i.e. 1-2 hours normally) plasma concentration drops rapidly. Thus, for the maintenance of plasma concentrations of flavonoids regular intake is required.

Certain classes of flavonoids such as flavonols and flavones etc. are normally glycosylated. The sugar can be substituted at any position on the compound and could be further replaced by an acid group e.g. malonic. The removal (by enzymes e.g. cosidases) of hydrophilic moiety (sugars) is typically required for passive diffusion through the intestinal wall, as a first step of metabolism. In the acidic conditions of the stomach, non-enzymatic deglycosylation does not occur (Gee et al., 1998).

It was proposed by De Eds in 1959 that flavonoids are readily absorbed and converted to a range of hydroxyaromatic acids which are quickly excreted in the urine. This suggests scission of larger flavonoids molecules into lower weight forms, in gastrointestinal tract before absorption. The metabolic transformation of flavonoids involves methylation of hydroxyl group, hydrogenation of side chains, dehyroxylation in the para-position and β-oxidation forming conjugates (Rice-Evans et al., 1995) determined by their chemical structure. Another potential factor affecting the uptake could be the interaction of flavonoids with protein species as flavonoids are multi-dentate ligands which have ability to bind simultaneously at different points on protein surface (Hagerman and Butler,

1981). Studies confirmed that various flavonoids readily autoxidise in chemical systems including myricetin, quercetin, delphinidin and querceitagetin drawing conclusion that adjacent tri or para di-hydroxyl groups bearing flavonoids, show significant rate of autoxidation producing superoxide and hydrogen peroxide (Hodnick et al., 1994). Studies confirm that some flavonoids exhibit pro-oxidant properties (in higher concentrations) in the presence of metal ions and stimulate hydroxyl radical production and DNA damage *in vitro*. The metal chelating potential of some flavonoids, depend on their precise structure and adjacency of their hydroxyl groups. Thus, there may be a possibility of them chelating metal ions and preventing metal catalysed development of initiating radical species (Aruoma et al., 1993). Higher concentrations of myricetin and gossypetin showed involvement in alteration of LDL through covalent modification of $apoB_{100}$ protein. These studies used relatively higher concentrations of the flavonoids (i.e. 100μM), which are unlikely to be achieved *in vivo* systems (Rankin et al., 1993).

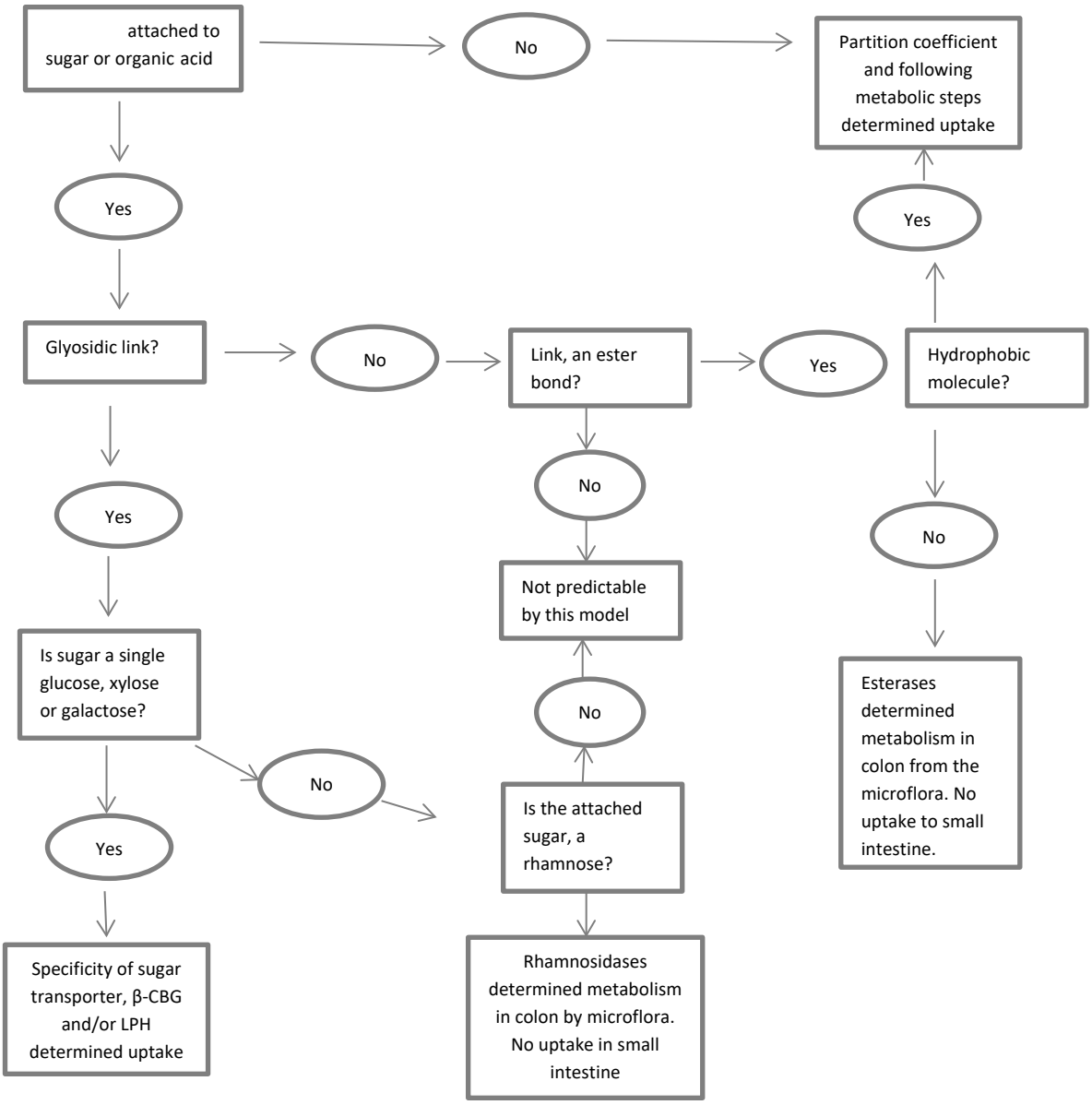


Figure 1.1a Absorption of flavonoids in human, based on *in vitro* and *in vivo* studies, a hypothesis (Panche, 2016)

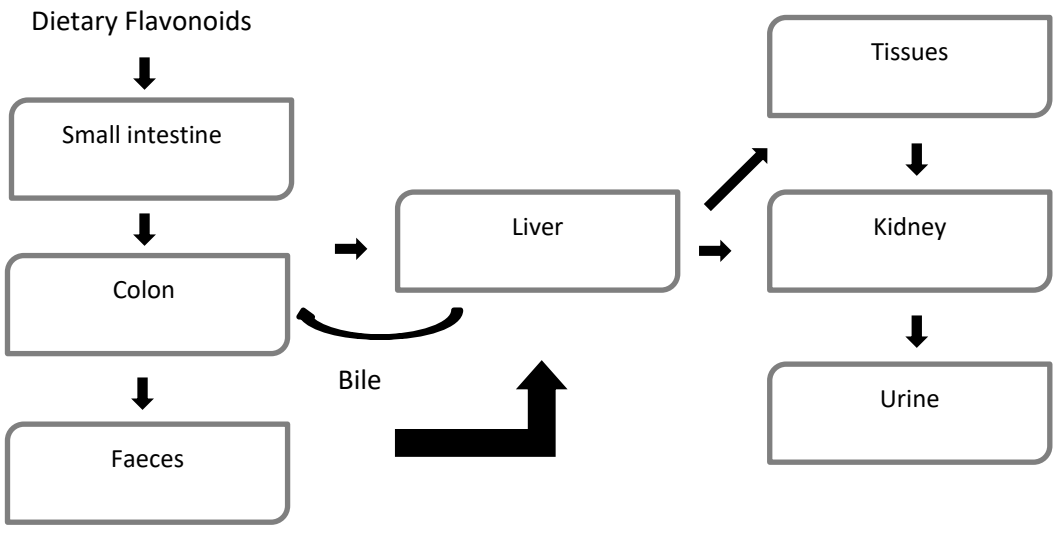


Figure 1.1b Potential routes of ingested flavonoids (Panche, 2016)

1.5.1 Flavonoids as antioxidant agents

Flavonoids are described as antioxidants (most abundant reducing agents in our diet), directly as free radical scavengers and indirectly by modulating intra-cellular pro and antioxidant enzymes but these compounds have the potential of performing dual actions and protect our body's tissues against oxidative damage. Thus depending on cell type, the applied stimulus and disease state, unpredictable results are possible when flavonoids interact with intracellular signalling pathways (Justino, 2010). For example they may provide protection against oxidative stress related diseases such as cancers and cardiovascular. The biological properties (bioavailability, interactions with cellular receptors and enzymes, antioxidant potential and others) of flavonoids depend on their chemical structure.

Flavonoids are regarded as antioxidants due to the ease at which an H atom can be abstracted from their molecule by a radical (e.g. ROS), producing a flavonoid radical which is more stable and less reactive than the original attacking radical. For a flavonoid to be an antioxidant, it has to fulfil two basic conditions: first, it can negatively affect the free radical-mediated oxidation or autoxidation when present in relative concentration to the substrate to be oxidised; second, the resultant radical should be stable through intramolecular hydrogen bonding by additional oxidation (Shahidi, 1992). Maximum radical scavenging effectiveness requires a C ring with a 3-OH group attached to 2-3 double bond and adjacent to 4-carbonyl (unsaturation). The di-hydroxyl groups adjacent to each other on B ring strongly influence the antioxidant activity of a flavonol whereas these groups at different positions are not that useful. Antioxidant activity does not enhance by adding more than two hydroxyl groups

in aqueous phase radicals. However, mono hydroxyl substituent containing a B ring is not an effective hydrogen donor and does reduce the activity (Pokorny, 1987). An extra hydroxyl group at position 5’ on B ring (i.e. on myricetin) does enhance the properties of flavonols in lipid systems (Pratt, 1990). Many studies have established that the position and extent of hydroxylation is a key to the antioxidant property of flavonoids (Furuno et al., 2002). The main determinants of radical scavenging potential are hence, summarized as; adjacent dihydroxylation on B ring (conferring higher stability to the radical form), a free hydroxyl group at position 5 and/or 3 of ring A and B, respectively, the carbonyl group at position 4 and 2-3 double bond in C ring (for electron delocalization from B ring) (Van Acker et al., 1996; Chobot and Hadacek, 2011).

Large numbers of studies have been piloted to investigate the beneficial effects of flavonoids. The ability to scavenge reactive oxygen species (ROS) shows the chemo preventative properties of flavonoids; however their pro-oxidant action could be a crucial process for their apoptosis-inducing and anticancer properties as ROS have the ability to mediate fragmentation of apoptotic deoxyribonucleic acid (DNA) (Anderson et al., 1998; Wasson et al., 2008).

1.5.2 Myricetin and its protective effects against various diseases

Myricetin belongs to a class of flavonoids, flavonols: bearing B-ring hydroxylation in the 3' and 4' positions. Myricetin has an extra hydroxyl group attached at position 5’ in B ring in contrast with other flavonols. It also has a hydroxyl group attached to position 3 with a double bond from positions 2-3 on C ring and at position 5 and 7 on A ring, as well as a carbonyl group at position 4. The unique structure (Figure 1.2) of myricetin determines its strong antioxidant property and other biological functions. The hydroxyl groups

adjacent to each other on B ring are responsible for conferring higher stability to the formed radical whereas the unsaturated C ring with carbonyl group at position 3 is crucial for electron localisation from the B ring. All these factors contribute to the maximum radical scavenging potential of myricetin (Pratt, 1990; Chobot and Hadacek, 2011).

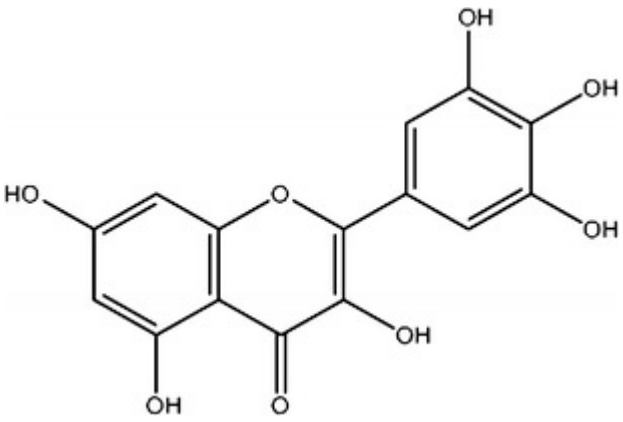


Figure 1.2 Structure of myricetin (Büchter et al., 2013)

The presence of a reactive oxygen species (ROS) i.e. •OH, $•O_2^-$ and H_2O_2 can cause oxidative stress to the biological molecules. Although, ROS are normally produced as by-products of cellular metabolism activities, their higher levels are potentially damaging to the cells, DNA, lipids and proteins could steadily lead to the development of various diseases. Myricetin presents various oxidative properties. It acts as an antioxidant by directly scavenging the free radical species (ROS), by chelating the intracellular metal ions (which eventually produce ROS) or by inducing intra-cellular antioxidant enzymes (Ross and Kasum, 2002). Myricetin can increase the positive effect of other antioxidants and is able to induce glutathione-S-tranferase (GSH) enzyme which protects cells against free radicals and peroxides.

Studies demonstrate that myricetin has the potential to act as a pro-oxidant due to its ability to autoxidise, depending on the substrate and environment, also

due to its tendency, to accelerate the production of hydroxyl radicals by reacting with ferrous or ferric (bound to EDTA) and H_2O_2. The ability of myricetin to act as an inhibitory agent against glutathione reductase (GR) also demonstrates its pro-oxidative characteristic (Ong and Khoo, 1997; Semwal et al., 2016).

Studies have shown that myricetin is very beneficial flavonoid antioxidant which has the ability to protect cells from hydrogen peroxide (H_2O_2) –induced damage, apoptosis and exhibits cyto-protective effects. It reduces adverse effects of H_2O_2 by decreasing the fragmentation of DNA, inhibiting the release of mitochondrial cytochrome C, increasing Bcl-2 up regulation and enhancing Bax down regulation. Myricetin is effective against both mitochondrial dependent and caspase dependent induced oxidative stress through regulation of different signalling pathways (Kang et al., 2010). Studies *(in vitro)* have proved that myricetin can directly lessen DNA damage caused by H_2O_2 in human lymphocytes, inhibits strands breakage and protects against oxidative base damage (Duthie et al., 1997). Myricetin triggered apoptosis in cisplatin (a cancer treatment drug used for the induction of apoptosis) resistant ovarian cancer cell lines, OVCAR-3 and A2780/CP70 through intrinsic (regulated by P53) and extrinsic pathways (Huang, 2015).

1.6 Food derived mutagens and carcinogens

A large number of human cancers, about 70-80%, are associated with lifestyle factors, of which 35-45% accounts for diet. Dietary factors which correlate strongly with cancer include low fibre, use of well-cooked food, high fat consumption, nitrite intake, less vitamin C usage, mycotoxins and alcohol (Doll and Peto 1981; Parkin et al., 2011). Heterocyclic amines (HCAs) comprise a group of compounds formed when meat is cooked and are considered to be

powerful bacterial genotoxins. HCAs are produced by cooking proteinaceous food, at very high temperature, allowing the reaction of creatinine, free amino acids and monosaccharides (Schut and Snyderwine, 1999; Sugimura et al., 2004). More than 20 carcinogenic/mutagenic HCAs have been isolated (Nagao et al., 1997; Wakabayashi et al., 1992). The main subclasses of HCAs found in the human diet include aminoimidazoazaarenes (AIA), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinolone(MeIQ),2-amino 3,8dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine(PhIP) (Schut and Snyderwine, 1999; Turesky and Marchand, 2011). Studies have shown these to be rodent carcinogens as well (Sugimura and Sato 1983; Sugimura et al., 2004). Activation of HCA to genotoxic compounds only implicates N-hydroxylation of exocyclic amines role whereas C-oxidation is just a purification reaction. Research shows that humans are exposed to HCAs through diet which are bioavailable and converted to genotoxic derivatives by CYP1A2 and can bind to DNA causing mutations but their association with diet related tumours development is not fully understood. Although a number of host drug-metabolizing enzymes have the ability to activate and detoxify HCA including CYP1A2, *N*-acetyltransferase, sulphotransferase, prolyl tRNA synthetase, phosphorylase and COX isomers (Wolz et al., 2000). The involvement of HCA in the etiology of human cancer is well evident, which has triggered several positive efforts to determine various exogenous and endogenous factors that transform health risks caused by HCA. The production of oxidative damage, in addition to the DNA adducts plays a significant part in the carcinogenic activity of food mutagens (Najefzadeh, 2009)

1.6.1 PhIP, a potential carcinogen

Diet is an important avoidable cause of cancer (WHO). Consumption of well-done cooked meat is associated with an increased risk of breast and other cancers. This association between meat preparation time and an elevated cancer risk is most likely due to an abundant formation of HCA. The most common HCA in the human diet is 2- amino-1-methyl-6 phenylimidazo [4,5-b] pyridine (PhIP) and it is also considered to be the most abundant HCA responsible for triggering different tumours in rats (Felton, 2004).

The oxidation of PhIP by cytochrome P450 (CYP1A2) forms an N-hydroxy derivative, which sustains successive sulphation or acetylation to produce a nitrenium ion free radical that covalently binds to guanine residues at the C8 position (Turesky, 2007). The consequent adduct is the main inducer of GC→TA transversions and toxic frameshift alterations of a guanine within a G-rich repetitive sequence (Gooderham, 2002). The formation of these mutations is the basis for the genotoxic effects of PhIP comprising DNA damage, chromosomal aberrations, chromatid exchange and micronuclei (Otsuka, 1996). A failure to correct these genomic faults can lead to cancer, especially when they occur in proto-oncogenes and tumour suppressor genes. Research shows oxidative stress as a possible causing factor of DNA damage caused by HCAs (Maeda et al., 1999; Murata et al., 1999; Gooderham, 2002).

1.7 Nanotechnology

Nanotechnology has been very important and useful in biomedical applications. This is a technique where designed nanostructures are assembled. Nano materials have been successfully used for different processes including protein characterization, bio sensing, drug delivery also for enzymes and biometric assemblies (Sun et al., 2014)

Nanotechnology is the systematic study of a specific system at the nano level (1-100nm). The US National Nanotech Initiative defines nanomedicine as those drugs with particle size between 1-100nm (Boisseau and Loubaton 2011). However most of the literature accepts that nanomedicine can be anything up to 500nm or even a 1micron (Martin 2006). The European Technology Platform on Nanomedicine describes nanomedicine as follows: 'Nanomedicine is defined as the application of nanotechnology to health. It exploits the improved and often novel physical, chemical, and biological properties of materials at the nanometric scale. Nanomedicine has potential impact on the prevention, early and reliable diagnosis and treatment of disease' (Boisseau and Loubaton 2011). It is worth noting that their definition eliminates the scale of the nanoparticles classified under nano medicine. In all cases submicron particles have been shown to exhibit a higher dissolution rate than those above 3 microns. Although particles with less than 100nm have a higher tendency to cross some biological membranes, it is worth noting that larger nanoparticles are likely to shrink in size when diluted in body fluid before reaching the size of action.

The maximum average bond lengths range at which molecules can manipulate at the molecular level in the pico metre range is 74 pico metres for H-H bond and 200 for C-I bond. But nanotechnology provides further opportunities for

material science research, medicine, biology and several other disciplines through manipulation of single atoms and molecules in a certain manner which fits into a specific application (Nie et al., 2007). Nanotechnology reduces the limitations and difficulties of many other techniques such as proteomics technology and help to retrieve fresh information from biological systems that were not otherwise possible with conventional methods. Several fundamental cellular structures comprising proteins, lipid polymer and carbohydrates have similar sizes to numerous nanostructures (Vo-Dinh, 2005). This similarity between nanostructures and biological molecules is very significant in design and manufacture of modern nano-assemblies that have crucial biotechnological and medical applications (Yang et al., 2015).

1.7.1 History of nanotechnology

Nanoparticles were introduced in the 9th century by artisans who used them for glittering effects or lustre on the surface of pottery. These NPs were produced by reacting copper and silver oxides with vinegar, salts, clay and ochre on fixed pottery and heated to 600^{0}C. Copper and silver migrated to the outer surface when the glaze would have been softening by the heat. Reformation of ions to metal form was prevented by maintaining low pressure which helped NPs to obtain optic effects. The lustre technique was developed by the Muslim world when Muslim men were not allowed to wear gold hence they achieved similar effects on ornaments without using gold. The first scientific definition of NPs was proposed in 1857 by Michael Faraday. The very initial thought about nanotechnology was delivered by an American physicist, Richard Feynman at the American Physical Society on December 1959. He defined nanotechnology as a process by which smaller atoms and molecules can be developed by

deploying larger ones (Taniguchi, 1974). Later on the term was described by Professor Norio Taniguchi in 1974 as a procedure of separation, consolidation and deformation of compounds using single atoms or molecules. In the beginning of 1980s, nanotechnology started development and with it came the discovery of the scanning tunnelling microscope (STM). This progress led to the Innovation of fullerenes and carbon nanotubes in 1985. Then in 2000, the United States National Nanotechnology Initiative was founded to organize Federal nanotechnology research and improvement (Guzman et al., 2006; Tinkle, 2010).

Nanotechnology is currently being utilised to tackle global water challenges to make water safe and purified (Mauter et al., 2018), in agricultural field to enhance livestock and crop productivity (Jasrotia et al., 2018), in cosmeceuticals (Kaul et al., 2018), in medicine field for effective drug delivery to treatment various diseases including cancer (Hu et al., 2018) and in storage of solar energy (Mohamed et al., 2018).

1.7.2 Properties of nanoparticles

NPs are in between the form of the bulk material and atomic structure of a compound. Bulk materials show persistent physio-chemical characteristics irrespective of their size, whereas NPs depict completely diverse properties (Hutter and Maysinger, 2010). NPs are agglomerated as they are held together by different kinetic forces including Van der Waals, electrostatic forces and sintered bonds. The solubility of particles is greatly affected by these forces between agglomerates, under various conditions (Allouni et al., 2009). As the size decreases to nanometre, the surface area to volume ratio increases. This enhances the diffusion capacity and reactivity, particularly at high temperatures.

Reduction in size lowers the melting temperature of the particles and changes various other catalytic properties as well (Buffat, 1976).

1.7.3 Applications of nanotechnology

Nanomedicine is a vast industry which utilizes the medical applications of nanotechnology. The nanosize particles of the drug maximise its bioavailability to the site of need, for a continued period of time. Thus the biggest application of nanomedicine is drug delivery. However there is a need to understand the toxicity of NPs to tackle current problems (Amiji, 2010).

NPs are very beneficial in cancer diagnosis, for example, in MRI imaging more detailed and higher contrast images can be produced of the cancer site due to size tuneable light emission (Kubik et al., 2005). Due to ineffective lymphatic drainage, NPs favourably accumulate at the tumour site because of their reduced size. This could be useful to consider NPs in future cancer treatments for targeting specific tumours and replacing the existing therapies (Leary et al., 2006). Scientists are considering the development of a multifunctional nanoparticle which could directly target tumour cells (Brown et al., 2010).

Other possible future medical uses of NPs include photo-dynamic therapy (Yamakoshi et al., 1999), surgery (Matteini et al., 2010) and cell repair etc. (Moghimi et al., 2005). However, commercially NPs are used in consumer products such as sunscreens, cosmetics, foodstuffs, outside furniture polishes, surface glaze and paint etc. (Parlini, 2008). Other applications of NPs include household use, nano-filtration, optics and in the textile industry (Bai et al., 2010).

1.7.4 Toxicity of nanoparticles

Although, NPs quickly agglomerate in the environment there still is the possibility of environmental and clinical risks due to their unpredictable activities particularly large surface area which makes them more reactive and easily diffusible through membrane barriers, allowing interaction with biological systems (Bharali and Mousa, 2010). NPs can enter our tissues through different routes i.e. breathing (Oberdorster, 2001), the digestive system (Jung et al., 2000) and perhaps through the skin (Kreilgaard, 2002).

Hence, in the modern era rapid and excessive use of engineered nano-particles in research and various fields requires emphasis on toxicological matters related to NPs exposure. NPs have the potential to cause DNA damage, and there is a strong association of DNA damage with mutations and then carcinogenesis, which is critical. Different mechanisms of NPs causing DNA damage could be due to the production of oxidative stress, direct interaction with DNA by disturbing spindle fibre plus other components or by inducing genome instability through epigenetic mechanisms. Some damage inducing properties of NPs include their size, agglomeration state, release of toxic metal ions and their capability to produce ROS. Hence detection of genotoxicity, induced by NPs is very important and crucial for reducing their effects. (Karlsson et al., 2015).

1.8 Genotoxicity produced by ROS induced Oxidative stress

Genotoxicity is described as a characteristic of chemical substances that causes harm to the DNA, destroys DNA structure, damages genetic material and segregation leading to mutations, which may cause serious consequences in the absence of effective repair mechanism, initiating mutagenesis which further leads to tumorigenesis (Magdolenova et al., 2012). Genotoxicity and

mutagenicity are often confused, since all genotoxic agents are not mutagen but all mutagens are genotoxic. Hence mutagenicity is a step further away from genotoxicity. Mutagens may cause DNA damage directly or indirectly. Cells naturally use their defence mechanism (i.e. enzymatic and non-enzymatic system) to fight against genotoxic substances and ROS. Antioxidant enzymes in the cell and antioxidant nanoparticles work together as a first line of defence against ROS and maintain their concentration at levels compatible with cell integrity (Schins, 2002). All direct genotoxic agents certainly cause DNA damage but the damage induced at lower levels can be potentially repaired by DNA repair processes (Jenkins et al., 2010).

ROS are oxygen containing ions and molecules which are normally produced as by-products of biological processes such as cellular metabolism. They play a vital role in inducing apoptosis under various physiological and pathological conditions. However, high ROS levels are damaging to cells, they cause oxidative stress leading to cellular malfunctioning and death.

It has been documented that the cellular production of ROS increases under oxidative stress in various conditions. If expression of genotoxic mutations is not prevented either by repair mechanisms or by apoptosis, damage may not be fixed continuing degenerative processes leading to mutagenesis and cancer (Moody and Hassan 1982). DNA by definition is the prime target of mutagenesis. Hence mutations in DNA may lead to initiation and development of cancer, as compromised integrity of genetic material has been known to cause tumorigenesis. Different genotoxic agents cause DNA damage by their respective mechanism of action. Some transition metals while in high-valent oxidation states interact with DNA to incur lesions leading to cancer while some

substances such as pyrrolizidine alkaloids (PAs) cause damage by producing DNA adducts, breaks, micronuclei, aberrations, cross linking and gene mutation etc. in vivo and in vitro. PAs are mutagenic and are responsible for causing cancer especially in the liver (Chen et al., 2010).

Genotoxic effects, if they do not immediately lead to death, can cause cancer. Severity of DNA damage is not the same among populations as among individuals differ in their ability to detoxify genotoxic agents. This leads to differences in occurrence of cancer among people. This ability comes from inherited polymorphisms of certain genes, important in activation of the chemical or due to variations in the efficiency of repair mechanisms of individuals (Bolognesi, 2003). Metabolism of some chemical substances leads to the generation of ROS, which is a possible system of genotoxicity.

1.9 DNA damage and repair mechanisms

Our body is composed of trillions of cells and every cell sustains thousands of lesions per day caused by various sources such as sunlight, chemicals, cellular metabolism and radiations etc. (Browner et al., 2004). Thus our cellular DNA constantly receives these insults which trigger DNA damage and gradually lead to DNA adducts (DNA segments bound to any carcinogen), interrupt gene expression, compromise genome integrity, mutations and structural alteration to the DNA. This damage could be either intrinsic (replication error, hydrolytic or ROS) or extrinsic (radiations, alkylation compounds, base analogues, intercalating agents). The mismatch repair system (MRS) is responsible for ensuring the accuracy of replication. Post-replication defects can only be corrected if the MRS recognises mismatch pair, determine the incorrect base, excises it and repairs the DNA. Most frequent hydrolytic damage includes

deamination when during replication, cytosine is converted to uracil and adenine is introduced (instead of guanine) at the opposite position. The most severe hydrolytic attack is when the purine base is totally removed from the DNA backbone (Branzei and Foiani, 2008). ROS (oxidising agents) present an important source of DNA damage, are not only produced by ionising radiations but also from cellular metabolism (Roy and Sil, 2012). Oxidation of guanine leads to 7, 8-dihydro-8-oxoguanine or 'oxoG', which readily pairs with adenine or cytosine. The type of mis-pairing promoted by oxoG would lead to a G:C→T:A transversion. Alkylation (by mutagens) transfers methyl or ethyl groups to either the base itself or to phosphate groups on the DNA backbone (Whitaker et al., 2017). Base analogues are almost identical in structure to genuine bases but are highly mutagenic (e.g. 5-bromouracil). Intercalating agents are compounds that are able to slide into the DNA backbone adjacent to purine or pyrimidine bases (e.g. ethidium bromide)

Our DNA is a very precious component; hence, our cells have evolved a variety of repair mechanisms for protecting it from assaults, to maintain our genome integrity and stability (Dexheimer, 2013). The cells initiate the DNA repair response which recognises, detects and mediates repair mechanisms by activating different pathways such as apoptosis, cell-cycle arrest or DNA repair. Accumulated DNA damage and impaired damage response could lead to the development of many conditions and diseases including cancer.

DNA damage could be repaired by the following mechanisms:

1.9.1 Base Excision Repair (BER)

Damaged bases are generally repaired via the 3 R's: Recognition, Removal and Replacement. In the case of BER an enzyme termed a glycosylase recognises

the damaged base, ‘snips’ it out of the DNA backbone and the resulting gap is filled in. Although excision repair is the most common mechanism for repairing damaged bases, a simpler mechanism also exists such as direct reversal ‘undoes’ the damage done to the base. For example, for direct reversal of alkylation events, an alkyl group is detected and removed by a DNA methyltransferase or glycosylase (Watson et al., 2004). Usually used for repair of single strand breaks (SSBs).

1.9.2 Nucleotide excision repair (NER)

NER does not recognise specific damaged bases rather it detects errors in the shape of the DNA helix. All the damage is repaired using the undamaged complementary strand of DNA (Wilson et al., 1997).

1.9.3 Homologous recombinant (HR)

Homologous recombination is very important process. In addition to repairing double strand breaks (DSBs) caused by Ionising radiation, homologous recombination is essential during meiosis. HR requires an identical sequence to be used as a template for the damaged chromosome repair through a sister chromatid which would be available in G2 (Watanabe et al., 2009).

1.9.4 Non-homologous end-joining (NHEJ)

Non-homologous end-joining (NHEJ) is a major DNA DSB repair system that a human cell can employ to repair DSBs. All proteins participating in this pathway possess significant mechanistic flexibility which allows NHEJ to produce various outcomes initiating from same ends (Srivastava and Raghavan, 2015). DNA Ligase IV together with cofactor XRCC4 act together to directly join the two broken DNA ends (Wilson et al., 1997; Gerodimos et al., 2017).

1.10 The cell cycle controls and cancer genetics

Cellular growth and division is a fundamental characteristic of all living organisms. The cell cycle starts from two daughter cells originated from a single parental cell and finishes when the daughter cells divide. The cycle consists of following phases: M phase; short span phase when nuclear division (mitosis) occurs followed by cytoplasmic division (cytokinesis), G1 phase; (longest) cellular growth phase where some cells differentiate further and some never escape from this phase rather enter G0 (resting phase), S phase; accounts for DNA synthesis, genome replication and checkpoints prone to DNA damage, G2 phase; rapid growth prior to M-phase with G2-M checkpoints (figure 1.3). The entire cell cycle machinery is regulated by and dependent on cyclin-dependent kinases (CdKs) either activation or inhibition (Hamel and Hanley-Hyde, 1997) and triggered by the action of growth factors. Cdks in G1 activate and stimulate a protein called retinoblastomas (Rb) to release a transcription factor (E2F) that then switches on the necessary genes to drive the cell into S-phase. Defects in Rb regulation are a common cause of cancer. Checkpoint mechanisms ensure the accomplishment of the previous phase before entering the next one.

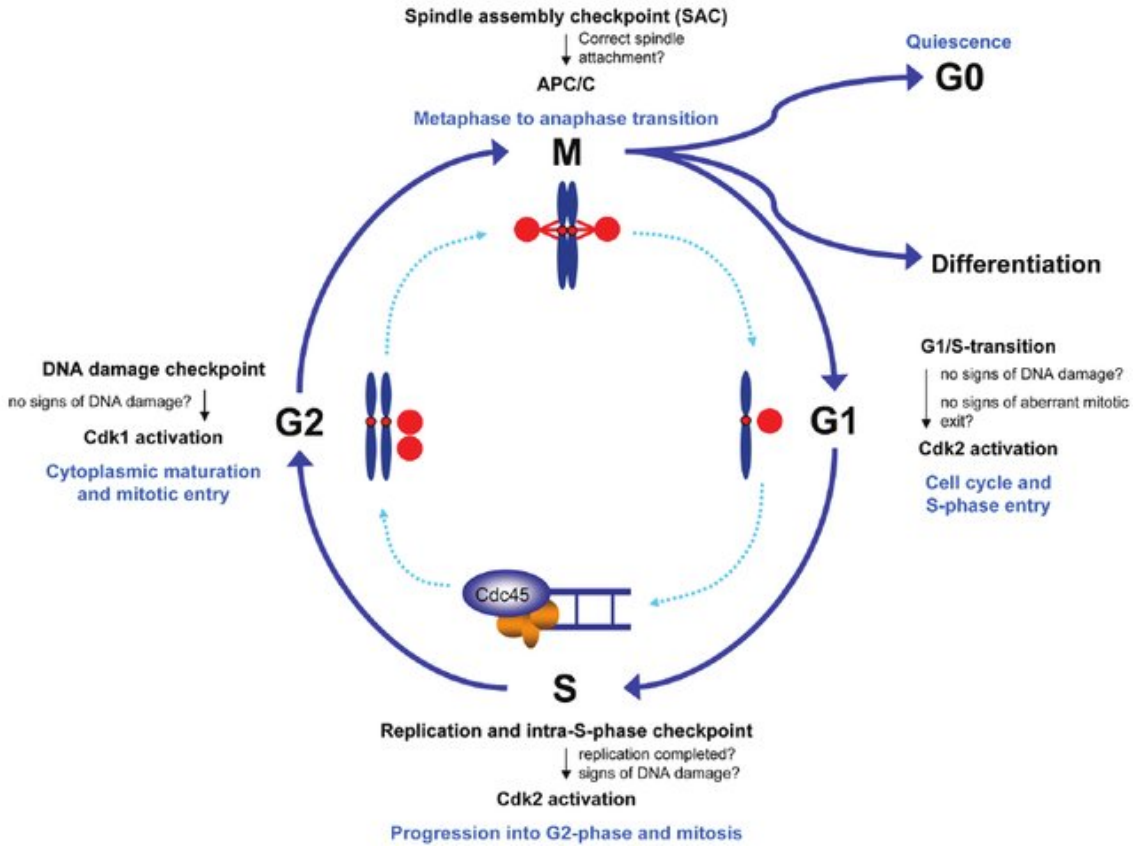


Figure 1.3 Cell cycle and its checkpoints (Schnerch et al., 2012)

Normally cell growth is a highly regulated process but when it is derailed, it leads to the production of uncontrolled cellular growth referred to as a tumour or neoplasm. These could be either benign (restricted to one point) or metastatic (spread around the body). Cancer cells are clonal which may be produced due to exposure to chemical substances which in turn cause DNA damage or due to chromosomal aberrations. Transformation (normal cell to tumour) occurs when a cell acquires a mutation and evades cell cycle check points i.e. G_1/S-phase (replication), G_2/M-phase (DNA damage check by P53 or PRb) and mitotic spindle (spindle formation) (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Malignant cells are generally defective at all these checkpoints. There are three main types of cancer genes; tumour suppressor genes, oncogenes and DNA repair genes. Tumour suppressor genes are also called anti-oncogenes; their function in normal cells is to regulate cellular

proliferation. Mutations in these genes lead to cancer development and loss of function (Lodish et al., 2000b). Oncogenes (cancer causing genes) activate cellular proliferation leading to differentiation and unregulated cellular growth (Ponder, 2001). They develop from proto-oncogenes which are responsible for regulation of normal cell growth processes. The normal function of DNA repair genes is to correct errors which occur when cells duplicate before cell division, whereas mutation in these genes results in failure of the repair system allowing successive mutations to accumulate.

1.10.1 P53 role in cell cycle regulation

P53 is a crucial gene, encoding 393 amino-acid proteins, arranged into various domains and plays a substantial role in cell cycle regulation. The fundamental domain (aa102-292) of P53 has been presented, responsible for sequence-specific DNA binding action to the protein (Wang et al., 1993). This particular domain encompasses the four reserved areas of the gene and the most important mutation hot-spots found in cancer-derived mutants (Pavletich et al., 1993). P53 also contains C-terminus and N terminal domain which concentrate on the majority of various post-transcriptional alteration sites that maintain regulation of its function (Appella and Anderson, 2001). P53 can incorporate various signalling pathways which are triggered by different stimuli including DNA damage, oncogene initiation or hypoxia. In such circumstances P53 stimulates different metabolic responses which ultimately lead to senescence, cell-cycle arrest, apoptosis, separation, DNA repair and inhibition of angiogenesis. The G1-S checkpoint on the cell cycle inhibits the replication of cells with damaged DNA and P53 plays a prominent role at this transition and arrests cellular growth there. It has been proposed that cell growth arrest (G1 arrest) is mediated by transcriptional regulation of p21 (inhibitor of CDKs

2,3,4,6), GADD45, Cdc25C and 14-3-3s dependant on P53 (Hermeking et al., 1997). Studies also show that Cdc25A initiates the delay in P53 dependent growth arrest at G1 which gives sufficient time for the cell to repair the damaged DNA (Nyberg et al., 2002). DNA repair can be triggered by P53 through the induction of p21, GADD45 and p48 protein expression (Hwang et al., 1999) and helps maintain genome integrity. Bax is amongst those P53 target genes which are involved in P53-induced apoptosis and are up-regulated (Shaw, 1996) but there are a few genes, down-regulated by P53 including Bcl 2 (anti-apoptotic gene) and cyclin A (a key protein in progression through S phase). The main responses of P53, cell-cycle arrest and apoptosis are usually based on stimulus and cell type. This is not fully understood that when and which pathway is activated by P53 first, though it is proposed that it could be dependent on affinity. Detecting low levels of DNA damage, it is possible that P53 upregulates high affinity genes and initiates cellular arrest. In contrast when stress levels cross the repair capacity then apoptotic genes might be activated (Inga et al., 2002). P53 also demonstrates transcriptional-independent activity in mitochondrial apoptosis (Mihara et al., 2003), though most of the studies have focused on its transcriptional related role. The role of P53 in S-phase checkpoints is proposed to stop the cells with incomplete DNA replication, from entering mitosis (Taylor et al., 1999). P53 plays a role in the maintenance of growth arrest at G2-M phase where various P53 targets also play a part (Shaw, 1996).

1.10.2 Mutant P53 (TP53) and cancer

P53 in its wild-form (WTP53) is the key tumour suppressor which provides protection against cancer. Missense mutation (substituting a single amino acid in P53 protein), commonly clustered in the DNA binding domain in mutant P53

(TP53) (P53 encoded gene) is the most common genetic damage observed in all tumour types and loss of wild-type function is a key process towards the cancer development (Olivier et al., 2010). As P53 is dominant negative, one mutated copy can corrupt the tetramer of whole protein. Loss of function results in the complete absence of P53 expression or production of mutant proteins which are unstable and truncated. Like all other tumour suppressors, the normal function of P53 has to be completely inactivated for a tumour to develop; hence, it needs two genetic lesions of the gene to occur, one on each allele in each cell, i.e. dominant negative. The resultant TP53 might depict diverse biological and biochemical characteristics, not present in its wild-type and could contribute towards the tumour developmental stages (Muller and Vousden, 2014). These properties and activities are often referred to as gain of function (GOF) manifesting dominant-negative impact over WTP53. A gradual rise in genome instability is a major hallmark of tumour progression demonstrated from increased mutations levels to deviations in chromosomal number and arrangement. TP53 can increase genome instability by disrupting the normal spindle formation checkpoint mechanism resulting in clusters of cells with polyploidy genomes (Gualberto et al., 1998). It can also affect the DNA repair by weakening the BER (Offer et al., 1999). A study has demonstrated that depletion of TP53 decreases tumourgenicity (both *in vitro* and *in vivo)*, anticancer drug resistance, and proliferation in a range of cancer cell lines. It also weakens the aggressiveness of cancers (Bossi et al., 2006). This gives an appealing explanation of the effects of TP53 on cancer progression. TP53 has the ability to make the cell susceptible to a raised resistance against various pro-apoptotic signals (Lotem and Sachs, 1995). Its anti-apoptosis action may

not only speed up the cancer development but also reduce the response to cancer therapies.

The anti-proliferative effects of the transforming growth factor β (TGF-β) on epithelial cells are supposed to be facilitated by WTP53 at the early stage of cancer (Cordenonsi et al., 2003). Hence, TP53 existence at this stage could override the inhibitory effect of TGF-β and if it influences after the proliferation stage, it could worsen the condition. Thus overexpressed TP53 could accelerate the tumour cells proliferation. Efficient GOF activity of TP53 depends on its higher levels in the affected cells and stability gained through cancer progression. As WTP53 is short lived and degraded mainly by Mdm2, evasion from Mdm2-mediated degradation is a key factor stabilizing TP53 (Oren and Rotter, 2010). TP53 is the most occurring mutated gene in all human cancer.

1.10.3 Mechanism of apoptosis and related genes

Apoptosis is programmed cell death. It occurs in multicellular organisms and is a crucial process to maintain genome stability. Excess of it causes atrophy whereas defective apoptosis gives rise to uncontrolled growth and various conditions including cancer. Induction of apoptosis in cancer cells may present a promising strategy against drug resistance as it was reported that decreased vulnerability of tumours to apoptosis was closely associated with drug resistance (Hall et al., 2008). It is activated by the protein caspases and derived through two pathways intrinsic (mitochondrial) and extrinsic (receptor-mediated). An intrinsic pathway is regulated by the Bcl-2 family proteins (pro; Bax, Bad and anti; Bcl-2, Bcl-xl etc.) which controls the mitochondrial membrane permeability and discharge of pro-apoptotic factors (Brunelle and Letai, 2009) whereas extrinsic pathways are regulated by tumour necrosis

factor-related apoptosis-inducing ligand (TRAIL) comprising Apo2L/TRAIL and associated receptors. The balance between pro and anti-apoptotic proteins, which is regulated by P53, decides whether a cell will undergo apoptosis or not. Loss of P53 correlates with high levels of anti and low levels of pro-apoptotic proteins (Kim et al 2014).

1.10.4 Role of Gamma-H2AX (γ-H2AX) in recognition and signalling of DNA double-strand breaks (DSBs)

Since the study of the DNA damage which leads to serious illnesses like cancer, is essential for understanding the underlying processes, the emergence of a rapid and sensitive method and biomarkers, needed to quantify the damage, has great potential to not only monitor the tumour response but also the effect of certain treatment on normal cells predicting any acute or long term consequences.

DSBs are the most damaging cellular lesions which, if left unrepaired could bring severe consequences for cellular homeostasis. It is believed that formation of a single DSB may be enough to disrupt the genome and cause cancer or cell death (Jackson, 2002). Hence, a system to locate and quantify these breaks is highly desirable. For this purpose, the natural protein function of the cell involved in repair processes could be used to locate DSBs under fluorescence microscopy.

Various factors are involved in DSB induction. Our cells are designed to activate a highly systematic mechanism called DNA damage response (DDR) in reaction to any kind of DNA damage, which detects and repairs it. The induction of DSBs triggers a process where a large number of chromatin protein, H2AX (a subtype of H2A), is phosphorylated forming γ -H2AX at the site of the break on location, serine 139 of H2AX, giving rise to a distinct γ-H2AX focus per DSB. This

phosphorylation plays a crucial role in DDR for gathering DNA repair proteins at damaged chromatin sites, also for the initiation of important checkpoint proteins which stops the cell cycle progression (Podhorecka, 2010). Histone H2AX is a substrate of various phosphoinositide 3-kinase-related protein kinases (PIKKs) such as ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) dependent on the origin of the DNA damage and timing, but ATM is considered as the main physiological mediator of H2AX phosphorylation in response to DSB formation. ATM is initiated by its auto-phosphorylation at Serine 1981 location, which separates the inactive ATM dimers into single protein molecules with improved kinase activity (Bakkenist and Kastan, 2003).

Visualisation and quantification of DNA DSBs can be achieved by fluorescent staining of nuclei for γ-H2AX foci, using specific antibodies. There is a direct correspondence between DSBs and γ-H2AX foci (Sedelnikova et al., 2002).

As lymphocytes can be easily taken from a donor's peripheral vein, use of γ-H2AX staining to detect DSBs in these cells is considered as an established method (Löbrich et al., 2005), as explained further in section 1.11.1.3.

Gamma-H2AX is a sensitive and primary indicator of DSBs *in vitro* and *in vivo*. It has proved beneficial for sensing low-levels of DNA damage. The use of γ-H2AX in cancer therapy is currently being investigated. As studies have shown that the H2AX gene location, 11q23, is commonly transformed in tumour cells which may bring a 3-fold rise in γ-H2AX development. As a marker for DNA damage, H2AX will be greater in transformed tumour cells due to mutated and unregulated cell cycles (Kuo and Yang, 2008).

1.11 Methods for the study of genotoxic effects

Genotoxicity assays are methods commonly used to mark substances that are able to react with nucleic acids at very low concentrations. This interaction between DNA and toxic agents could result in chromosomal aberrations and mutations in DNA structure. This has the ability to affect the accuracy of the message, leading to reversible changes in the cell. Such DNA impairment could result in harmful consequences e.g. establishment of a disease, altered heritable features, decreased reproductive capacity and increased mortality (Cavas et al., 2014). There are a number of methods for assessing DNA damage that have been used to determine agents with genotoxic ability. The biological effects resulting from DNA damage include micronuclei, chromosomal structural changes and mutations. The single cell gel electrophoresis (SCGE) or the Comet assay is a useful and reliable approach for calculating this damage and has been extensively used in various studies conducted in our laboratory (Anderson et al., 1996; Anderson et al., 1997; Anderson et al., 1998a; Anderson et al., 1998b; Anderson et al., 1999; Anderson et al., 2003; Anderson, 2005).

1.11.1 The Comet Assay

1.11.1.1 Development and principles of the Comet assay

A micro gel electrophoresis technique for assessing DNA damage at single cell level was first developed by Ostling and Johanson in 1984. But due to use of neutral conditions for the procedure, the general utility of the assay was limited. Then Singh et al., (1988) formulated a micro gel electrophoresis method for detection of DNA damage in individual cells, under alkaline conditions (pH >13). This design of assay massively enhanced sensitivity for determining genotoxic

substances because most of the genotoxic agents activate a greater magnitude of SSB than DSB. A pH >13 is expected to maximize the expression of alkali labile sites (ALS) as SSBs. Since presentation of this assay at pH>13, its applications and uses have greatly increased. It has been used in various research areas including human and environmental biomonitoring, genetic toxicology and DNA repair processes.

The attributes of the Comet assay include its rapidness, simplicity, low cost and high sensitivity. Its advantages also include assessing very low levels of damage, less quantity of cells needed per sample, conduction of studies using relatively smaller amounts of test substances. The Comet assay is a standard procedure for calculating DNA genomic damage. On a microscope slide, cells fixed in agarose are lysed to liberate DNA forming nucleoids which comprise supercoiled rings of DNA connected to a nuclear matrix. Structures similar to comets are formed by electrophoresis towards the anode using ph >13 (Singh et al., 1988). Then alkali is neutralized followed by DNA staining with ethidium bromide or SYBR green. Finally observed under a fluorescence microscope where higher damage is shown by lengthy intense tails and less damage by relatively shorter tails. The word comet identifies the DNA migration pattern of individual cell, developed by this assay.

1.11.1.2 The Comet assay in genotoxicology

Studies have proved that complex associations of different antioxidants and various modifying agents with ROS related DNA damage inducing substances and their reprotoxic or genotoxic effects can be assessed in all types of eukaryotic cells including human lymphocytes using the Comet assay. According to the systemic review by Sponchiado in 2015 the single cell gel

electrophoresis (SCGE) or the Comet assay *in vivo* and *in vitro* is the 3rd most often used assay for genotoxicity assessment of herbal extracts. In humans it is used to determine genetic damage with the aim of accessing human exposure to genotoxic agents due to several factors including lifestyle and environment. It has been used for DNA repair studies and environmental biomonitoring (Faust et al., 2004). Genotoxicity of NPs has been examined by this assay for the last decade and it has been confirmed suitable for measuring their toxicity (Karlsson et al., 2015) (Magdelenova et al., 2012). The Comet assay is very suitable for use with lymphocytes because the DNA damage of somatic cells can potentially lead to cancer development (Gopalan et al., 2011).

1.11.1.3 Lymphocytes, as surrogate cells in genotoxic studies

Vulnerability to cancer depends on several determinants including sensitivity of the genome, exposure to genotoxins and probable efficiency of the DNA repairing system (Collins 2004). Lymphocytes are considered as an appropriate choice to examine genome sensitivity; this is because the sub-populations of lymphocytes have long lives and have the ability to retain genetic mutations induced by a mutagen for more than 40 years (Anderson et al., 2014).

As genotoxins cover a major percentage (>90%) of human carcinogens, use of lymphocytes is recommended by the World Health Organisation International Programme for Chemical Safety (WHO/IPCS), for detecting genotoxicity (WHO, 2000). Peripheral blood lymphocytes are easy to obtain for sampling and large numbers of them circulate around the body in the blood which makes them easy target for genotoxins (Albertini et al., 2000). Research suggests that cytokines and chemokines involved in tumour development impair lymphocyte functionality. Lymphocyte genomic damage is further enhanced when

inflammatory mechanisms directly affect cancer development by inducing mutations (Ben-Baruch, 2006). Chemokines are involved in regulation of various cellular processes in WBCs including lymphocytes via initiation of G-proteins (Guanine nucleotide binding proteins) and downstream receptor kinases and are important regulators of WBCs trafficking. Their participation in affecting the activities of other cell types and their expression in cancer development processes is well evident. Particular chemokines have been shown to be involved in recruitment of WBCs to tumour sites (Singh et al., 2011). This shows that the ability and function of the leukocytes including lymphocytes is somehow connected to cancer development processes. Therefore in the current study we used the lymphocytes as model cells from pre-cancerous and MM patients to investigate the effects of MYR B and MYR N.

As lymphocytes form a major cellular part of the adaptive immune response, the DNA damage and repair biomarkers could potentially provide information about disease status and condition in other cell types. For instance, DSBs are most deleterious types of DNA lesions which if left unrepaired could possibly cause mutations leading to cancer development and cell death (Jackson, 2002). Their detection in lymphocytes could provide a track and extent of DNA damage and repair in other cell types.

The circulation of lymphocytes throughout the body provides them best exposure to genotoxic chemicals thus lymphocytes reflect an overall state of the organism. Their long half-life, ability to be stimulated once thawed from frozen conditions and ability to repair the induced damage make them better surrogate cells to be used in human monitoring and genotoxicity studies than other WBCs ((Bausinger and Speit 2016).

1.11.2 The cytokinesis-block micronucleus (CBMN) assay

1.11.2.1 Improvement and principle

Micronuclei were assigned various names, terminologies and description in the past. These structures were constantly found after cells were exposed to radiation and it was assumed that micronuclei (MNi) develop from acentric fragments which at later phase of mitosis, are extruded from two daughter nuclei (Kirsch-Volders et al., 2003). Micronuclei were discovered as a biomarker for cytogenetic damage by Evans et al in 1959, when they compared effectiveness of gamma rays to that of neutrons in Vicia faba roots. Countryman and Heddle in 1976, for the first time described the development of the micronucleus assay in human lymphocytes. But major flaws were observed in this assay by Michael Fenech in its then current form, due to fact that the observed micronucleus frequency was dependent on two things i.e. ratio of lymphocytes that gave a response to mitogen stimulation and the number of divisions which took place before harvesting during the culture period. He predicted that this method would only be fully optimal when a way to identify once divided cells was established. He further found that the short-lived binucleated stage in telophase is the only point where once divided cells can be identified. The MN frequency could decrease with repeated cell divisions and hence comparison between MN frequencies and dividing cell populations could not be accessed. For this reason, cytokinesis needed to be blocked to stop cells completing cellular division after completing nuclear division. Cytochalasins are inhibitors of cytokinesis; they do this by stopping the polymerisation of actin into microfilaments which are essential for cytokinesis. Cytochalasin B is the most

efficient form that could inhibit cytokinesis in lymphocytes and other mammalian cells (Fenech, 2009).

Micronuclei are formed as a result of lesions at the DNA or chromosomal level or at the protein level which are involved in the segregation of chromosomes e.g. tubulin. Their formation normally requires meiotic or mitotic division.

Validity for the in vitro MN test for *in vitro* genotoxicity had been delayed due to limited understanding of mechanisms involved in induction of micronuclei and inadequate procedures to calculate cell proliferation and MN induction. But the combination of the cytokinesis block assay with immunochemical labelling of kinetochores, made it easier to identify major systems involved in MN induction, formation of double-stranded breaks (resulting in micronuclei with acentric fragments) and a compromised mitotic apparatus (leading to micronuclei with entire chromosomes). Specific aspects of the current protocol for the MN assay were developed and agreed upon at The Washington '2nd International Workshop on Genotoxicity Testing' (25-26 March 1999) on following topics comprising option of cells, assessment of micronuclei, slide preparation, use of cytochalasin B, toxicity, treatment times and number of doses.

The Human MicronNucleus (HUMN) project was aimed at establishing the application and understanding of the lymphocyte cytokinesis block micronucleus assay (CBMN). One of the fundamental aims of this project was to determine methodological variables and scoring MN to minimize their confounding effects, enabling better accuracy of the assay for determining genotoxic events. Therefore the reliability of the assay for comparing DNA damage rates increases. Also exposure conditions that induce increase in MN can be identified (Fenech et al., 2003).

1.11.2.2 Advantages

The MN assay is considered to be highly reliable, quick and a suitable method to determine a wide spectrum of DNA damage at basal level especially for mutagenicity assessment (Hovhannisyan, 2010). According to a systemic review (Sponchiado et al., 2015), the MN assay was the most frequently used method for evaluating genotoxicity of herbal extracts. Scoring has been made very simple and there are vast applications of the MN test *(in vitro*) in various cell types which have made it an accessible tool to determine cytogenetic damage. At present the CBMN method is the most widely used procedure for calculating MN frequency in human lymphocytes, and for determining the fate of a once-divided cell after chemical exposure (figure 1.4). CBMN can be used to score MN and other DNA biomarkers that are observed in binucleated cells (i.e. nucleoplasmic bridges and nuclear buds) and cells experiencing death either by apoptosis or necrosis are also counted. A positive association shown by various studies between MN, nuclear buds and nucleoplasmic bridges indicated these genomic instability biomarkers are mechanically linked to each other.

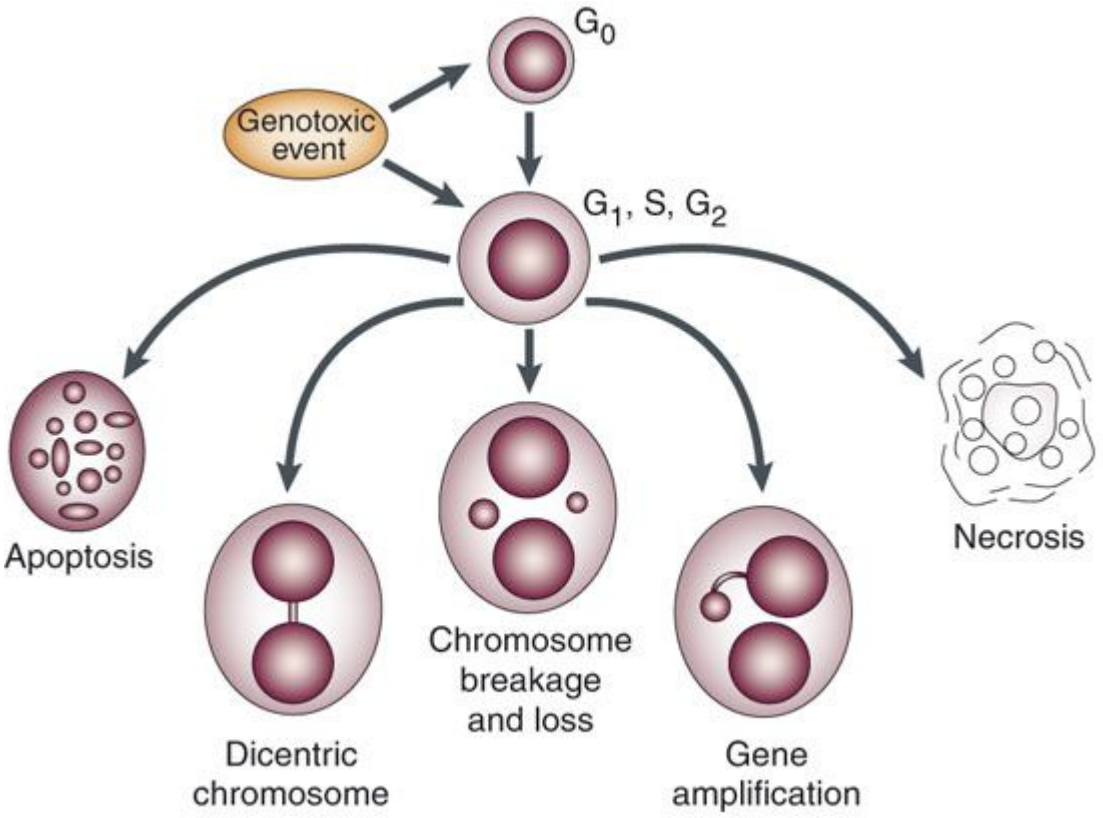


Figure 1.4 Possible fates of cykinesis-blocked cultured cells after exposure to chemical (cytotoxic/genotoxic) agents (Fenech, 2007)

1.11.3 Western blotting

Western blotting, also called protein immunoblot is a widely used technique in cellular and molecular biology, immunogenetics, biochemistry and other disciplines of biology, for protein detection and to quantify the protein expression. It enables researchers to identify particular proteins from a complex mixture of various proteins, extracted from cells. The technique is comprised of three parts to complete the task i.e. parting by size based on the molecular weight and type of proteins using gel electrophoresis, then blotting to a solid matrix producing a band for each protein and finally, visualising/marking target protein using correct primary and secondary antibodies. Unbound antibodies are washed off as specific antibodies bind only to corresponding proteins. Thus, only one band should be visible for each protein while developing the film. The

thickness of the band corresponds to the quantity of protein present in the sample which could be indicated using a standard. Western blotting could detect as low as 1ng of proteins due to its high resolution gel electrophoresis, strong specificity and greater sensitivity.

1.11.4 Real Time-PCR

Polymerase chain reaction (PCR) is an abundantly used technique in biomedical studies which enables quick and easy production of a large quantity of DNA from relatively smaller starting material. It amplifies a single or few copies of DNA to millions of copies of a specific DNA sequence. The method was developed by Kary Mullis in 1983 who later on won the Nobel Prize for this innovative invention in 1993. This easy, economic and reliable concept of replicating a focused DNA segment is widely applicable in various fields of science and probably is the most commonly used technique in molecular biology (Bartlett and Stirling, 2003). PCR Is simply ‘targeted’ DNA replication. DNA polymerases need a primer to provide a 3-OH group for DNA synthesis, and by having a ‘sense’ primer and an ‘antisense’ primer it is possible to specifically produce DNA in between the two primers (amplification). DNA polymerases synthesise a complementary strand in a 5’ to 3’ direction using one of the strands as a template. By using 2 primers, that are complementary to the two strands, both strands act as templates and are copied. Most PCR methods rely on thermal cycling which involves exposure of reactants to repeated heating and cooling cycles comprising three basic steps: Denaturation (melts the duplex into ssDNA), annealing (primers base-pair with ssDNA) and extension (DNA synthesis from primers). For denaturation the PCR reaction is heated to 94°C for 30 s to 2 mins to physically separate the DNA double helix

then followed by annealing at between 45°C to 72°C for 30 s where two DNA strands become a template for polymerase for selective amplification of target DNA. This temperature depends on the sequence of the primers and is determined by experiment (one temp at a time or some machines produce a temp gradient). Then extension at 72 or 78°C, depending on the heat stable DNA polymerase (Saiki et al., 1985). PCR represents an efficient *in vitro* cloning system that can produce as well as alter the defined DNA fragments in most simplified automatic reactions. PCR is extensively used in various applications including medical, infectious diseases diagnosis, forensics, DNA isolation, DNA amplification and quantification and research field etc. It is a powerful research tool bearing many advantages as being simple, rapid and a highly sensitive method producing billions of copies of target product.

1.11.5 Immunofluorescence (IF)/immunocytochemistry (ICC)

ICC/IF is widely used technique for light microscopy to stain specific proteins and antigens and visualise the cellular view of spacial and temporal dynamics of various metabolic processes (Bennett et al., 2009). It uses particular mono/polyclonal primary antibodies with fluorescent secondary antibodies against various proteins and utilizes fluorescent dyes to highlight the interaction and is visualised under epifluorescence microscope, allowing the naked eye to quantify the cells with DNA damage.

1.12 Aims and Objectives

Although myricetin is a well-researched antioxidant flavonoid which has tremendous health beneficiary effects against various types of cancers and cardiovascular diseases however, there is no evidence of research about its

effects on lymphocytes from pre-cancerous and MM patients. Therefore this study is conducted to determine the genoprotective and genotoxic effects of myricetin bulk (MYR B) and myricetin nano (MYR N) in lymphocytes from pre-cancerous and MM patients compared to those from healthy individuals. These aims will be achieved through following objectives:

- To compare the protective effects of myricetin bulk & nano forms on peripheral blood lymphocytes from healthy individuals and pre-cancerous patients. This will be achieved through assessing cytotoxicity using the MTT assay and DNA damage using the Comet & micronucleus assays as well as evaluation of p53 and ATM pathway.
- To investigate the *in vitro* protective role of MYR B and MYR N against ROS induced oxidative damage in lymphocytes from healthy individuals and pre-cancerous blood patients. This will be achieved by measuring cytotoxicity using the MTT assay, DNA damage caused by H_2O_2 in the Comet assay, the change in intracellular ROS and GSH levels, and assessment of DSB formation using immunocytochemistry.
- To determine the modulating effect of MYR B (10μM) and MYR N (20μM) on PhIP-induced DNA damage in lymphocytes from healthy individuals and pre-cancerous patients using the Comet and micronucleus assays to determine genotoxicity and DNA damage, and through studying P53, Bcl-2 and ATR pathways.
- To study the effect of MYR B (10μM) and MYR N (20μM) in lymphocytes from myeloma cancer patients compared to those from healthy individuals and investigate causative molecular mechanisms and pathways. This will be achieved through studying DNA damage using the Comet assay and cytotoxicity using the MTT assay. In addition analysis of P53, Bcl-2 and

Bax expression at the gene and protein level, and monitoring of ROS levels will also be carried out.

Chapter 2: Materials and Methods

2.1 Materials

All the chemicals, reagents and other materials used in various techniques in current project are listed in the table 2.1 below.

Chemicals, reagents, media, kits and antibodies	Suppliers	CAS number
Dimethyl sulphoxide DMSO	BDH, Poole, UK	67-68-5
Ethidium bromide	Sigma-Aldrich, UK	1239-45-8
Ethylenediaminetetraacetic acid	Sigma-Aldrich, UK	6381-92-6
Hydrogen peroxide	Sigma-Aldrich, UK	7722-84-1
Low melting point agarose	Invitrogen, UK	39346-81-1
Normal melting point (NMP) agarose	Invitrogen, UK	9012-36-6
PBS phosphate buffer saline	Sigma-Aldrich UK	79382
RPMI medium 1640 (with 2m M L-glutamine and 25 m M HEPES)	Invitrogen, UK	11875-093
Rothwell Dark RPMI 1640 medium with L-glutamine	Sigma-Aldrich UK	N/A
Sodium Chloride	BDH, Poole, UK	7647-14-5
Sodium Hydroxide	BDH, Poole, UK	1310-73-2
Triton X-100	BDH, Poole, UK	9002-93-1
Trizma base	Sigma-Aldrich, UK	77-86-1
Tween 20	Sigma-Aldrich, UK	9005-64-5
Trypan blue 0.4%	Sigma Aldrich, UK	72-57-1
MTT dye	Fisher Scientific, UK	M6494
Acetic acid	VWR, UK	72-31-15
Cytochalasin B	Sigma-Aldrich UK	14930-96-2
DPX mountant for histology	Sigma-Aldrich UK	44581
Disodium hydrogen phosphate (Na2HPO4)	VWR	7558-79-4
Ethanol	Sigma , UK	64-17-5
Formaldehyde	Sigma-Aldrich UK	50-00-0
Glacial acetic acid	Fisher Scientific , UK	64-19-7
Giemsa Stain improved R66 solution Gurr	VWR	51811-82-6
Mitomycin C	Sigma-Aldrich UK	50-07-7
Methanol	Fisher Scientific , UK	67-56-1
Phytohemagglutinin (PHA)	Invitrogen, Ltd	9008-97-3
Potassium chloride	VWR	7447-40-7
Penicillin-Streptomycin	Invitrogen Ltd	11528876
Sodium dihydrogen phosphate (NaH2PO4)	VWR	7558-80-7
Ammonium persulfate (APS)	Sigma Aldrich, UK	A1924
Anti-Mutant p53 antibody [Y5] Rabbit monoclonal	Abcam, UK	Ab32049
Anti-Bcl-2 antibody [E17] Rabbit monoclonal	Abcam, UK	Ab32124
Anti-Bax antibody [E63] Rabbit monoclonal	Abcam, UK	Ab32503
Acrylamide/Bis Acrylamide solution 30%	Sigma Aldrich, UK	A3699
Bovine serum albumin (BSA)	Sigma Aldrich, UK	9048-46-8
Bromophenol blue	Sigma Aldrich, UK	115-39-9
Bradford dye reagent	Bio Rad, UK	5000205

Donkey Anti-Rabbit IgG H&L (HRP) Polyclonal antibody	Abcam, UK	Ab205722
ECL 1, 2	BioRad, UK	1705060
Foetal bovine serum (FBS)	Sigma Aldrich, UK	9014-81-7
GADPH primary antibody, Rabbit monoclonal	Abcam, UK	Ab8245
Glycine	VWR, UK	56-40-6
Halt TM Protease inhibitor cocktails (100X)	Fisher scientific, UK	78430
Low Fluorescence Western Membrane (PVDF)	Abcam, UK	Ab133411
Laemmli sample buffer 2X concentrate	Sigma Aldrich, UK	S3401
Lymphoprep	Stem cell Technologies, UK	07851
2-Mercaptoethanol	Sigma Aldrich, York, UK	60-24-2
Non-fat dried milk (NFDM) powder	Tesco, Ltd, UK	3024250
Precision Plus Protein™ Dual Colour Standards,. Ladder	Bio Rad, UK	1610374S
Ripa lysis buffer	Fisher scientific, UK	89900
SDS	Sigma Aldrich, UK	75-05-8
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma Aldrich, UK	110-18-9
DCFDA / H2DCFDA - Cellular Reactive Oxygen Species Detection Assay Kit	Abcam, UK	Ab113851
96-well black plates	PerkinElmerTM	6005660
GSH/GSSG Ratio detection assay kit (fluorometric green)	Abcam, UK	Ab138881
AurumTM Total RNA mini kit	Bio-Rad, UK	732-6890
iScriptTM c DNA synthesis kit	Bio-Rad, UK	170-8890
Fast SyBRR green master mix	Thermo Fisher, UK	4385612
Nuclease free water	Qiagen, UK	129114
Primers	Sigma Aldrich, UK	-
MicroAmp® Fast Optical 96-Well Reaction Plate, 0.1 mL	Thermo Fisher, UK	4347907
Goat Anti-Mouse IgG H&L (Alexa Fluor® 488), polyclonal secondary	Abcam, UK	ab150113
Dapi	Invitrogen, UK	D1306
Gold antifade reagent with Dapi	Invitrogen, UK	8961
Anti-gamma H2A.X (phospho S139) antibody [9F3] Mouse monoclonal	Abcam, UK	ab26350

Table 2.1 Materials along with their suppliers and CAS numbers

2.2 General Methods

2.2.1 Ethical approval

The current project which involves the use of human peripheral lymphocytes has been granted ethical approval by Leeds East Ethics Committee (Reference No.:12/YH/0464) and the University of Bradford's Sub-Committee for Ethics in Research involving healthy Human Subjects (Reference No.: 0405/8). All peripheral blood samples were collected after informed consent from patients

and healthy individuals. The research support and governance office of Bradford Teaching Hospitals NHS Foundation also agreed the research (REDA number 1202).

2.2.2 Blood collection

All the basic information (age, gender, medicines and food supplement intake, lifestyle factors, diet etc.) was obtained through the questionnaire filled in by the patients and healthy individuals (Please refer to Appendix 1, 2 and 3 for the forms used to collect the data).

Blood samples from healthy volunteers were collected in the phlebotomy room, University of Bradford, UK by trained phlebotomists. Whereas blood samples from patient groups were kindly collected and provided by the hospital staff at Bradford Royal Infirmary (BRI), Haematology Department, Bradford, UK.

Whole blood was collected by venepuncture in anti-coagulant tubes from the healthy individuals and the patient group (see table 2.2 & 2.3). Samples were diluted with1:1 ratio of RPMI 1640 medium (Sigma Aldrich) complemented with 10% dimethyl sulphoxide (DMSO), aliquoted into labelled Eppendorf tubes and instantly stored at -80^{0}C to be used for the Comet assay and fresh blood was used to isolate lymphocytes for rest of the techniques used in the current study.

No	Age	Ethnicity	Gender	Smoking history	Family history
1	48	CAUCASIAN	M	NO	NONE
2	28	CAUCASIAN	M	NO	NONE
3	27	AFRICAN	M	YES	NONE
4	38	CAUCASIAN	M	NO	NONE
5	60	CAUCASIAN	F	NO	NONE
6	40	ARAB	M	NO	NONE
7	45	CAUCASIAN	M	YES	NONE
8	55	CAUCASIAN	M	NO	NONE
9	35	CAUCASIAN	M	YES	NONE
10	25	CAUCASIAN	M	NO	NONE

11	44	ASIAN	M	YES	NONE
12	28	CAUCASIAN	M	NO	NONE
13	23	CAUCASIAN	F	NO	NONE
14	27	CAUCASIAN	M	NO	KIDNEY CANCER
15	33	ARAB	M	YES	NONE
16	47	ASIAN	M	YES	NONE
17	28	CAUCASIAN	M	NO	NONE
18	42	ASIAN	M	NO	NONE
19	48	ASIAN	M	NO	NONE
20	60	ASIAN	M	YES	NONE
21	24	ASIAN	M	NO	NONE
22	34	ASIAN	M	NO	NONE
23	34	CAUCASIAN	F	YES	NONE
24	34	ASAIN	M	NO	NONE
25	59	CAUCASIAN	F	YES	NONE
26	28	ASIAN	M	YES	NONE
27	61	CAUCASIAN	F	NO	NONE
28	36	CAUCASIAN	F	NO	NONE
29	52	CAUCASAIN	F	NO	NONE

Table 2.2 Brief information about the healthy blood samples

No	**Age**	**Ethnicity**	**Gender**	**Smoking history**	**Family history**	**Medical condition**
1	79	CAUCASIAN	M	NO	NONE	MGUS, COPD, MONOCLONAL B CELL LYMPHOCYTOSIS
2	80	CAUCASIAN	F	NO	NONE-	MGUS
3	78	CAUCASIAN	M	NO	NONE-	MGUS
4	73	-	F	YES	CANCER	MARGINAL ZONE LYMPHOMA
5	56	-	F	YES	LEUKAEMIA & BRAIN TUMOUR	MGUS
6	77	-	M	NO	NONE	MGUS
7	75	-	M	NO	NONE	MGUS
8	80	-	M	NO	CANCER POSITIVE	MGUS
9	81	CAUCASIAN	F	NO	BOWEL& STOMACH	MGUS
10	63	CAUCASIAN	M	YES	NONE	MGUS
11	72	CAUCASIAN	M	NO	NONE	ARTHRITIS
12	55	CAUCASIAN	M	NO	NONE	MGUS
13	83	CAUCASIAN	M	NO	NONE	MGUS, BENIGN PROSTATIC HYPERPLASIA
14	63	CAUCASIAN	M	YES	NONE	MGUS
15	74	CAUCASIAN	M	NO	NONE	MGUS COPD
16	63	CAUCASIAN	F	YES	ARTHRITIS	MGUS, COPD

17	66	CAUCASIAN	F	NO	BREAST CANCER	MGUS
18	52	-	M	YES	NONE	MGUS
19	62	-	F	YES	LYMPHOMA & BRAIN TUMOUR	MARGINAL ZONE LYMPHOMA
20	63	-	M	YES	NONE	MGUS
21	-	CAUCASIAN	M	NO	NONE	MGUS
22	74	CAUCASIAN	F	NO	LUNG CANCER COPD	MGUS
23	83	CAUCASIAN	M	-	NONE	MGUS
24	60	ASIAN	F	NO	NONE	MGUS
25	62	-	M	NO	NONE	MGUS
26	51	-	F	NO	NONE	MGUS
27	74	-	M	NO	NONE	MGUS
28	75	-	F	NO	NONE	MGUS +TYPE II DIABETES
29	64	CAUCASIAN	M	NO	NONE	MARGINAL ZONE LYMPHOMA
30	55	CAUCASIAN	F	NO	NONE	MULTIPLE MYELOMA
31	56	CAUCASIAN	M	NO	PANCREATIC CACNER	MULTIPLE MYELOMA
32						
33						
34	84	CAUCASIAN	M	NO	NONE	MANTLE CELL LYMPHOMA
35	79	CAUCASIAN	M	NO	BREAST CANCER	MULTIPLE MYELOMA
36	68	CAUCASIAN	F	NO	NONE	MGUS
37	77	CAUCASIAN	F	NO	OVARIAN AND BREAST CANCER	MULTIPLE MYELOMA
38	70	CAUCASIAN	M	NO	NONE	MULTIPLE MYELOMA
39	50	ASIAN	F	NO	NONE	MGUS
40	87	CAUCASIAN	M	NO	NONE	MULTIPLE MYELOMA
41	61	CAUCASIAN	M	NO	TESTICULAR AND BREAST CANCER	MGUS
42	75	CAUCASIAN	F	NO	MASTECTOMY	MGUS
43	69	CAUCASIAN	M	NO	STOMACH AND LUNG	MGUS

Table 2.3 Brief information about the patient samples (MGUS stands for monoclonal gammopathy of undetermined significance, COPD for chronic obstructuve pulmonary diseases)

2.2.3 Preparation of myricetin Nano-particles (NPs), myricetin bulk and PhIP

Myricetin was purchased from Fisher Scientific, UK (529-44-2) as a powder. Suspensions of myricetin bulk and nanoparticles were made using 7% (w/w) solid loads of myricetin in a medium comprising of hydroxypropyl methylcellulose (HPMC) (0.5 % w/w), sodium lauryl sulphate (SLS) (0.1% w/w),

ethanol (0.8% w/w), polyvinylpyrrolidone (PVP) K-30 (0.5% w/w) and purified water. PhIP was prepared in double distilled water. The suspensions were transferred to an amber glass bottles and stored at 4^0C for the research duration.

2.2.4 Microfluidic Procedure

The nanoparticles of myricetin were prepared using a Y shaped microfluidic reactor in a precipitation technique. Five mls of ethanol containing the drug at a concentration of 5mg/ml was injected in one side of the channel and 10ml of water from other side at a rate of 30ml/hr and 60ml/hr, respectively. The liquid was then collected in a 50ml beaker, being stirred with a magnetic stirrer and all the above mentioned contents were added. The contents were stirred for another 10 mins after removing the precipitate.

2.2.5 Particle size of mricetin bulk and nanoparticles

The mean particle sizes of myricetin nanoparticles in the stock solutions were measured using a Zetasizer Nano ZS-90 Model ZEN3600 (Malvern Instruments Ltd, UK) by Photon Correlation Spectroscopy. Measurements were taken at room temperature.

2.2.6 Transmission Electron Microscope (TEM) analysis of myricetin particles

2.2.6.1 Morphology and visualization of myricetin bulk and NPs

To visualise the individual particles and their shapes, transmission electron microscopy TEM Tecnai 12, (FEI Company, Netherlands) was used. The photographs were captured at different magnifications operated at 5 to 150K.

2.2.6.2 Preparation of TEM sample grids

Equipment: Filter paper, glass petri dish, parafilm, self-closing tweezers, slass or plastic pipette, 3 tumblers, doubled distilled water (DD-H_2O), timer, desiccator (silica gel), grids and uranyl acetate (TAAB Laboratories, UK).

Method: Grids were placed onto the filter papers and a drop of each sample, in a correct dilution ratio was applied to them individually and left for 10 mins to dry. The grids were sequentially cleaned in the 3 tumblers with DD-H_2O and placed on filter papers to dry. Parafilm was prepared in the petri dish to contain any uranyl acetate spills and grids were placed in uranyl acetate for 2mins. Grids were again sequentially cleaned in 3 tumblers of DD-H_2O then placed on filter papers to dry overnight followed by visualization of particle sizes of myricetin bulk and nano at different magnifications using TEM.

2.2.7 Stability of myricetin bulk and NPs

The stability of the particles for both forms of myricetin was assessed by checking their particle size and the difference was less than 1%. Hence, these were considered stable to be used for the study. The suspensions were also sonicated for 10 mins before each use to avoid sedimentation and control aggregation.

2.2.8 Myricetin and PhIP concentrations

In the current study two forms of myricetin (NPs, bulk) and one form of PhIP (Toronto Research chemicals INC, Canada. A617000) (bulk) was used. The concentrations of myricetin bulk (MYR B) and NPs (MYR N) forms, used for in this research study were 10µM and 20 µM respectively determined by concentration dependent studies as non-genotoxic concentrations. The

concentration of PhIP used throughout the study was 100 µM and was determined by dose response curve. (Data shown in chapter 3 and chapter 5)

2.2.9 Isolation of lymphocytes from whole blood

Whole blood was diluted with 0.9% saline (by dissolving 9g of NaCl per 1000ml water) in 1:1 dilution and mixed thoroughly. Then diluted blood was overlaid on top of pre-filled lymphoprep (Invitrogen, UK) in a falcon tube (1:0.5 dilutions) without mixing the two. The tube was centrifuged at 800g for 20mins at room temperature (RT). The lymphocyte layer was carefully transferred to a saline solution and centrifuged for another 15mins at 500g. All the supernatant was tipped off and cells were suspended in RPMI-1640 (Sigma Aldrich, UK) media.

2.2.10 Cell density calculations (Haemocytometer)

Fifty µl of 0.4% trypan blue was added to 350µl RPMI media and 100µl of isolated lymphocyte stock together. Ten µl of this suspension was placed onto each chamber of a haemocytometer. Four big squares (within 4 small squares in each) were counted for cell numbers using following formulae:

Dilution factor = final volume/volume of cells stock,

Cell density =(No. of live cells per big square/volume of square per ml) x dilution factor.

2.2.11 Cell viability using trypan blue exclusion

One ml suspension of isolated lymphocytes was added to a micro centrifuge tube and treated with 10µl of test chemical followed by incubation at 37^0C for the desired time. After the incubation time was over the tubes were micro centrifuged for 5mins at 4000rpm. The supernatant was removed and the

remaining 50 µl pellet was re-suspended by pipetting up and down several times; 10 µl of this was transferred to an empty Eppendorf ® tube. Then 10 µl of Trypan blue (0.4%) was added to the tube containing cells, mixed well and left for 30 secs to settle. Then 10 µl of this suspension was transferred to a slide. A small coverslip was placed on top and observed under the light microscope. Live cells appeared transparent and the dead ones appeared blue. 100 cells were counted for each slide to check viability percentage.

2.2.12 MTT assay

To evaluate cytotoxicity and cell survival rate, the MTT assay was used as it is a standard sensitive, simple, reliable and qualitative method to determine cytotoxicity, using less material. To start the experiment 10-20,000 cells/well (lymphocytes isolated from whole blood) were seeded in a 96-well plate and incubated at 37^0C overnight in the cell culture incubator to let cells firmly attach to the bottom of the plate. The next day, treatment solutions were added to each well at the desired concentrations. In addition to test wells, one set included just culture media without any cells (blank) and one set included culture media with cells but not exposed to chemical compounds (negative control). The plate was left in the incubator for different periods (1, 24, 48 hrs). After the exposure time was over, the medium was replaced by fresh medium and 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (5mg/ml of PBS) was added to each well and incubated for 4 hours at same conditions of incubation. After this the medium was gently aspirated from each well and 200 µl of DMSO was added to dissolve the formazan crystals (chromogenic products of the reduction of tetrazolium salts by dehydrogenases and reductases). The contents of each well were gently mixed by pipetting

DMSO up and down 4-5 times. Absorbance was read and recorded after 10mins at 590nm using a plate reader.

2.2.13 The Comet Assay

2.2.13.1 Cell treatment

A volume of 10µl of treatment solutions, 100 µl of whole blood and 890 µl suspension of RPMI medium were added in Eppendorf ® tubes with a final volume of 1000 µl and incubated at 37^0C for 30 min. The tubes were centrifuged for 3 min at 3000rpm and then 900 µl of the supernatant was removed and discarded. The remaining cell pellet was re-suspended and mixed with 100 µl of 0.5% low melting point (LMP) agarose (made in PBS w/v); then 100 µl of this cell mixture was placed on a dry slide pre-coated with 1% normal melting point (NMP) agarose (1% w/v in ddH_2O) and spread out using suitable glass coverslips. The agarose was allowed to set on ice for few minutes.

2.2.13.2 Lysis

Once the agarose layers were set, the cover slips were removed and slides were incubated in freshly prepared lysis solution (89% lysis buffer, 10% DMSO, 1% triton X-100) overnight. Lysis buffer is a detergent containing high salt solution (2.5 M NaCl, 100 mM EDTA and 10 mM Trizma base at pH 10).

2.2.13.3 Electrophoresis

The slides were kept horizontally on the gel electrophoresis tank and incubated in fresh cold alkaline electrophoresis solution (10mls of 200mM EDTA & 60mls of 10M NaOH mixed in 1930mls of ddH_2O at pH > 13) for 30 min at 4°C for

unwinding the DNA then electrophoresis was run at same conditions for another 30 min at about 300mA current and 25 Volts.

2.2.13.4 Neutralisation

The slides were removed from the electrophoresis solution and washed in neutralisation buffer (0.4 M Trizma base in ddH_2O at pH 7.5) three times at 5min intervals.

2.2.13.5 Staining

The DNA was visualized by staining the slides with 60 µl of ethidium bromide (EB) (20µg/ml) and covered with the cover slips.

2.2.13.6 Scoring of Slides

All slides were correctly coded according to different treatment groups and 100 cells were scored per slide using a fluorescence microscope equipped with the CCD camera and computer system. Data were produced measuring two parameters: Olive tail moment (OTM) and % Tail DNA by using Komet 6 software, Kinetic imaging (Andor Technology Ltd, Belfast, UK) (Magnification: X20, Image calibration: X81: Y^*1).

2.2.13.7 Statistical Analysis

The normality of data obtained was tested using the Kolmogorov – Smirnov test. The data was analysed by one-way ANOVA to determine the significance figure. All values $P < 0.05$ were considered significant. Statistical analysis was performed using Graph Pad Prism 7.02.

2.2.14 Micronucleus Assay

Fresh blood was obtained from 5 patients and 5 healthy volunteers. The samples were collected in heparinised tubes and used for the cell cultures.

2.2.14.1 Cell culture medium preparation

A fume hood was used for all the culture steps under sterile conditions. The basic culture medium (RPMI 1640 with 25mM HEPES and L-Glutamine, 1% penicillin-streptomycin and 15% foetal bovine serum(FBS)) 4.5ml, was transferred into each 25cm^3 corning vented cap culture flask and was stored at -20°C until needed. Prior to use, the flasks were equilibrated at 37°C (with 5% CO_2) for at least 30min.

2.2.14.2 Culturing blood and Treatment

To start the culture, 130 µl of phytohaemagglutinin (PHA) and 370 µl of fresh blood was added to the flasks containing the basic medium at 0 h. The flasks were gently shaken to mix the contents and incubated for 24 h at 37°C in the presence of 5% CO_2. At 24 h, 50 µl of each chemical was added to the cultures. Plain medium was used for the negative control (NC), 0.4 µM mitomycin C was added to the positive control (PC) and flasks were further incubated for 20 h under the same conditions. After 44 h of treatment, 30 µl of cytochalasin B (cyt-B) (1mg/ml) was added to each culture to block cytokinesis. The flasks were left for incubation for another 28 hrs.

The flasks were removed from incubation at 72 hrs and contents shifted to 15 ml falcon tubes. Sterile conditions were no longer required. The tubes were centrifuged at 800rpm for 8 min, the supernatant was removed from each culture until 500 µl remained, using a vacuum pump. To give cells the hypotonic shock, 5ml of cold 90mM KCL was added to each tube while being stirred

gently on a vortex then incubated for 15mins at 4°C to allow the cells to relax. The tubes were centrifuged and the supernatant was discarded until 500 µl remained.

2.2.14.3 Fixation steps

A 5ml freshly prepared Carnoy's solution (3 parts methanol and 1 part glacial acetic acid) was added to each tube drop by drop while being gently mixed on a vortex tailed by 3 drops of formaldehyde (38%) and stirred well by a Pasteur pipette (Fisher Scientific) . The cell suspensions were centrifuged at 800rpm for 8 min, the supernatant was discarded until 500 µl remained and cells were re-suspended by patting the tubes. The fixation step was repeated twice without the addition of formaldehyde and tubes were left overnight at 4°C.

2.2.14.4 Slide preparation and staining

The next day, tubes were centrifuged for 8min; the supernatant was removed and discarded until approximately 100 µl remained. Then about 200-600 µl of Cornoy's solution was added depending on the cell density and pellet size. A volume of 20µl of cell suspension was placed twice on pre-labelled frosted glass slides (centre left and centre right) and allowed to air-dry. For each treatment group four slides were prepared and the cell density was checked using a phase contrast microscope (Nikon Eclipse Ti-S) and left overnight to dry. The following day, slides were stained with Giemsa solution (5% Giemsa solution made in phosphate buffer at pH 6.8) for 20min and smoothly washed in water for 2min and left to dry. Three drops of DPX mountant for histology were placed and cover slips 24 x 50 mm^2 (VWR) were mounted on to the prepared slides and dried overnight at room temperature.

2.2.14.5 Micronuclei (MNi) Scoring

To determine the frequency of MN, 1000 cells were scored for each treatment group under 40 x magnifications using a light microscope, according to criteria characterized by Fenech (2007) Table 2.4. The nuclear division index (NDI) was calculated using the frequency of MN in 1000 binucleated (BiNC) lymphocytes.

NDI = (M1 + 2(M2) + 3(M3) / N

Where M1-M3 represent number of cells with 1-3 nuclei and N = the total number of viable cells scored (i.e. 1000 cells per treatment group). The NDI shows the effect of a compound on the cell cycle of viable cells. Its value normally lies between 1.0 and 2.0 but could vary and be even greater than 2.0 if the cells were able to complete more than one nuclear divisions and MultiNC number increased while MoNC number decreased.

2.2.14.6 Statistical Analysis

Each experiment was repeated 3 times in 3 individuals and values are presented as the mean and standard deviation (SD). The differences between various treatment groups were determined by performing normality checks. Data were analysed using One-way ANOVA and t-test on Graph Pad Prism 7.02

Scoring criteria

Criteria for scoring viable mono-, bi- and multinucleated cells

- Mono-, bi and multinucleated cells are viable cells with an intact cytoplasm and normal nucleus morphology containing one, two and three or more nuclei, respectively.

Criteria for scoring apoptotic cells

- Apoptotic lymphocytes are cells undergoing programmed cell death. They have the following characteristics: Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear membranes.
- Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane.
- Staining intensity of the nucleus, nuclear fragments and cytoplasm in both kinds of apoptotic cell is usually greater than that of viable cells.

Criteria for scoring necrotic cells

- Early necrotic cells can be identified by their pale cytoplasm, the presence of numerous vacuoles (mainly in the cytoplasm and sometimes in the nucleus), damaged cytoplasmic membrane and a fairly intact nucleus
- Late necrotic cells exhibit loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary.
- Staining intensity of the nucleus and cytoplasm in both types of necrotic cell is usually less than that observed in viable cells.

Criteria for selecting BN cells suitable for scoring MNi, NPBs and NBUDs

- The cells should be binucleated.
- The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
- The two nuclei within a BN cell may be attached by a nucleoplasmic bridge, which is no wider than 1/4th of the nuclear diameter.
- The two main nuclei in a BN cell may touch but ideally should not overlap each other.

Criteria for scoring micronuclei

- The diameter of MNi in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.
- MNi are non-refractile and they can therefore be readily distinguished from artifact such as staining particles. MNi are not linked or connected to the main nuclei.
- MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense .

Criteria for scoring nucleoplasmic bridges

- The width of an NPB may vary considerably but usually does not exceed 1/4th of the diameter of the nuclei within the cell.
- NPBs should also have the same staining characteristics as the main nuclei.
- On rare occasions, more than one NPB may be observed within one binucleated cell.
- A binucleated cell with an NPB may contain one or more MNi.
- BN cells with one or more NPBs and no MNi may also be observed.

Criteria for scoring nuclear buds

- NBUDs are similar to MNi in appearance with the exception that they are connected with the nucleus via a bridge that can be slightly narrower than the diameter of the bud or by a much thinner bridge depending on the stage of the extrusion process.
- NBUDs usually have the same staining intensity as MNi

Calculation of nuclear division index (NDI)

- The NDI provides a measure of the proliferative status of the viable cell fraction. It is therefore an indicator of cytostatic effects and, in the case of lymphocytes,
- It is also a measure of mitogenic response, which is useful as a biomarker of immune function.
- Score 500 viable cells to determine the frequency of cells with 1, 2, 3 or 4 nuclei, and calculate the NDI using the formula NDI = $(M_1 + 2M_2 + 3M_3 + 4M_4)/N$, where M_1–M_4 represent the number of cells with 1–4 nuclei and N is the total number of viable cells

scored (excluding necrotic and apoptotic cells). • The NDI is a useful parameter for comparing the mitogenic response of lymphocytes and cytostatic effects of agents examined in the assay. • The lowest NDI value possible is 1.0, which occurs if all of the viable cells have failed to divide during the cytokinesis-block period and is therefore all mononucleated. • If all viable cells completed one nuclear division and are therefore all binucleated, the NDI value is 2.0. • An NDI value can only be greater than 2.0 if a substantial proportion of viable cells have completed more than one nuclear division during the cytokinesis-block phase and therefore contain more than two nuclei.

Table 2.4 MN scoring criteria adapted from Fenech 2007

2.2.15 Western Blotting

2.2.15.1 Cell culture

Isolated lymphocytes (10^6) were placed in 6 well plates for each treatment group, RPMI medium supplemented with 15% foetal bovine serum (FBS) and 1% penicillin streptomycin was added to the cells producing the total volume of 5ml. Then 130µl of Phytohaemagglutinin (PHA) was added to the wells and incubated at 37^0C for 24 hrs in the presence of 5% CO_2. After the incubation time was over, the medium was exchanged with fresh medium and cells were treated with chemicals for 24hrs.

2.2.15.2 Cell lysis for protein extraction

(All steps were done on ice in the tissue culture hood)

Culture medium was carefully removed from each well and cells were washed twice with 1ml ice cold phosphate buffered saline (PBS) while being mixed gently. PBS was discarded and 150 µl lysis buffer supplemented with 15µl of fresh protease inhibitor cocktail was added to the cells. Then cells were scraped and transferred to cold Eppendorf ® tubes, vortexed and centrifuged for a few seconds and made ready for measuring the protein concentration.

2.2.15.3 Protein quantification assay with Bio-Rad Bradford assay

Five dilutions of bovine serum albumin (BSA) were prepared in PBS as follows and used as standards:

(2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml)

5µl of each sample and standard was pipetted into 96-well plates in triplicates. 250µl of Bio-Rad dye reagent was added to each well and incubated for 30mins. The absorbance was measured by a spectrophotometer using wavelength of 595nm with a microplate reader. The resulting absorbance value was used to quantify the protein concentration.

2.1.15.4 Sample preparation

Using the formulae: concentration = mass/volume, the volume of protein extract was determined to ensure the same weight in each well. 10µl of Laemmli sample buffer was added to each sample and the volume in each lane was equalized. A very minute amount of bromophenol blue was added to each sample (to aid in observing the dye front while the gel is running) and was thoroughly mixed. The samples were boiled at 100^0C for 5mins prior to loading.

2.2.15.5 Preparation of gels and solutions:

Separating gel: 5ml of distilled water (dH_2O) was taken in a universal tube and added in it was 6.67ml of 30% acrylamide/bis-acrylamide solution. Then 4ml of Tris (pH8.8) was added to the solution. It was further mixed in 160µl of 10% SDS followed by the addition of 16 µl of TEMED. Immediately prior to pouring the gel, was added 160 µl of 10% ammonium persulfate (APS) and mixed well.

Stacking gel: 5.3ml of ddH_2O was taken to begin with and 2ml of 30% acrylamide/bis-acrylamide solution was added to it. Then 2.5ml of 0.5M Tris (pH6.8) and 100 µl of 10% SDS were mixed in the solution. When ready to use, 10 µl of TEMED was added and immediately before pouring the gel, 100 µl of APS was mixed in.

APS 10%: Made up fresh each day by adding 100mg of APS powder in 1ml of distilled water.

Running buffer: Tris base (3g), glycine (14.4g) and SDS (1g) were dissolved on a magnetic stirrer to prepare 1 litre of running buffer in distilled water (dH_2O).

Blotting or transfer buffer: Tris base (3g) and glycine (14.4g) was mixed in 800ml of dH_2O. Methanol (200ml) was added to the solution to get a final volume of 1litre. Blotting buffer has to be made freshly for each use.

*TBS-T (Tris Base Saline – T)***:** To prepare 1 litre of TBST, NaCl (8.78g), Tris base (2.42g) and Tween (300µl) was added to dH_2O and pH was set to 7.4 while being mixed on a magnetic stirrer.

2.2.15.6 Protein polyacrylamide gel electrophoresis (PAGE)

It is a powerful analytical method used to characterize individual or multiple proteins in a complex sample. Mini-PROTEAN® Tetra Handcast Systems with

Power Pack™ Basic (Bio Rad, UK) was used to run the electrophoresis. To start the procedure, glass plates were cleaned with wet tissue to remove dirt residues and assembled with spacers in the rack assuring that there is no leakage. The separating gel was poured to about 1cm below the wells of comb avoiding any air bubbles and allowed to dry for 30mins. Once the separating gel was dried, the stacking gel was carefully poured on top and combs were

inserted. The gel was left for 15-30mins to settle. Glass plates with hardened gel were positioned into the gel holder assembly and immersed into the tank filled with cold running buffer. Combs were removed in the tank so that wells get quickly filled with running buffer. Protein samples were vortexed and 10-30µl of samples was carefully loaded into each well. Pre-stained protein ladder (5µl) was loaded as a control in the first lane to keep the track of protein migration of samples. A lid was placed on the tank and electrophoresis was run at 120V for 90mins.

2.2.15.7 Protein transfer to PVDF membrane

PVDF membrane was activated by placing it in methanol for 30secs and then in water for 30secs without touching the membrane with fingers. The cassette was assembled in the following order from the white side: sponge, 2 filter papers (cut to the size of the gel), PVDF membrane, resolving gel, 2 filter papers, sponge and black cover. Each component was soaked in transfer buffer before assembling to keep the membrane moist. Air bubbles were removed by rolling a pen across the second layer of sponge. The cassette was closed and placed in the tank already prepared with blotting buffer and ice pack. (It was made sure that black was to black and red to clear as gel runs from cathode to anode). Gel was run using power pack at 120V for 90mins.

2.2.15.8 Blocking the membrane

To prevent the non-specific binding of antibodies to the membrane and avoid a high background problem, the membrane was blocked as follows. The blocking buffer was prepared by dissolving 5% non-fat dried milk (NFDM) or 5% bovine serum albumin (BSA) in TBS-T and filtered twice. After electrophoresis was over the membrane was carefully taken to a square petri dish with 15ml of

blocking buffer and left on the rotator overnight in the cold room for blocking or blocked for 2hours at room temperature. The membrane was washed for 3 times with TBS-T (5 mins each).

2.2.15.9 Primary antibody preparation and incubation

The primary antibody (7.5µl) was added to 15ml of TBS-T with 5% BSA in a plate (1:2000). The membrane was inserted in the primary antibody dilution and left overnight on the cold room shaker. Then it was washed for 3times at room temperature with TBS-T (5mins each).

2.2.15.10 Secondary antibody preparation and incubation

7.5µl of secondary antibody was added to 15ml of TBS-T with 5% BSA in a plate (1:2000). The membrane was inserted and put on a shaker for 1 hour at room temperature while being covered with a foil. After the incubation time was over, the membrane was washed 3 times for 5mins each with TBS-T.

2.2.15.11 Visualisation

The membrane was incubated with equal volume of ECL solution 1 and 2 for 90secs before being taken to the developing cassette and visualized for bands using G-Box SYNGENE system and GeneSys software. Images were captured and saved to be analysed later.

2.2.15.12 Re-probing the membrane

The membrane was washed once with dH_2O and then 3 times with TBST-T (for 5mins each), blocked for 1 hour at RT and incubated with primary antibody overnight on a shaker in the cold room. The next day the membrane was washed again for 15mins and incubated with secondary antibody for 1 hour

while shaking at RT in the dark. It was washed after incubated and then visualised with the steps described above.

2.2.15.13 Statistical analysis

All the data were analysed using image j software to determine the protein expression levels and normalised against the internal gene, GAPDH. Each experiment was repeated 3 times in isolated lymphocytes from 3 individuals each from healthy and patient groups and values are presented as the mean and standard deviation (SD). The differences between the various treatment groups were determined by performing normality checks. Data were analysed using One-way ANOVA and the t-test on Graph Pad Prism 7.02.

2.2.16 Cellular ROS Detection Assay

(All steps to be carried out in the fume hood) Isolated lymphocytes supplemented with complete medium (RPMI 1640 no phenol red with 1% penicillin-streptomycin and 15% foetal bovine serum) were grown in a 96-well plate overnight. The next day the media was removed and cells were washed with 1X buffer (provided with the kit) then new media along with treatments was added and incubated for 1 hour at 37^0C in the presence of 5% CO_2. Once incubation was over the medium was again removed and cells were washed with 1X buffer following the addition of DCFDA dye into each well and incubated again for 45mins under the same conditions. Then dye was replaced by new media and fluorescence was measured at 485/535nm using Promega Glomax explorer version 2.4. Data were analysed using the t-test on Graph Pad Prism 7.02.

2.2.17 GSH/GSSG Ratio Detection Assay

(All step to be carried out in the fume hood and on ice) Isolated lymphocytes (10,000-100,000/well), supplemented with complete medium (RPMI medium along with with 15% foetal bovine serum (FBS) and 1% penicillin streptomycin) were harvested overnight in 6-well plate. The following day the cells were treated with chemicals for 1 hour. Then the media was carefully removed and cells were washed with cold PBS and re-suspended in 100µl of cold lysis buffer supplemented with 10µl of protease inhibitors. Cells were thoroughly mixed by pipetting and centrifuged at 400g for 5mins to remove insoluble. The supernatant (sample) was collected and kept on ice for further use. 50µl of each GSH and GSSG standards (kit components) were added to 96-well plates in duplicates. 50µl of sample was also added in duplicate corresponding to each standard. For GSH detection, 50µl of the GSH assay mixture (GAM) (provided with the kit) was added to each GSH standard and sample. For GSSG detection, total GSH assay mixture (TGAM) was added to each GSSG standard and sample. Incubated for 60mins at RT in the dark and then fluorescence was measured at 490/520nm using Promega Glumax explorer version 2.4. Data were analysed using t-test on Graph Pad Prism 7.02.

2.2.18 Real Time Polymerase chain reactions (q PCR)

2.2.18.1 Cell culturing

Isolated lymphocyte suspension (2 x 10^6 cells/ml) was incubated in 6-well plates with chemicals for 24 hrs at 37^0C in the presence of 5% CO_2.

2.3.18.2 RNA extraction (using kit)

According to the manufacturer's instruction, cells were rinsed with PBS and transferred to 2ml centrifuge tubes then centrifuged at 400g for 3 mins. The supernatant was discarded and 350µl of lysis buffer was added to the palette while mixing thoroughly to lyse the cells. Then 350µl of ethanol (70%) was added in the tubes and the whole suspension was pipetted in the RNA binding column (inserted into a 2ml capless wash tube) and centrifuged for 3 mins. The supernatant was discarded and 700µl of low stringency wash solution was added to the binding column, centrifuged again and exultant was discarded. 80µl of diluted DNase I was pipetted to the membrane stack on the bottom of each column and incubated at RT for 15mins to allow digestion. Washing steps were carried out again; first with high stringency solution followed by a lower one. After centrifugation for 3mins, the binding column was transferred to a new tube and 40µl of the elution buffer was added onto it and further centrifuged for 2mins to elute the total RNA.

2.2.18.3 RNA concentration and quality

The RNA concentration and A260/A280 ratio (absorbance at these values) was determined using IMPLEN, Nano photometer P330. The reading for blank (RNA diluent) and samples was calculated. The RNA A260/A280 ratio within 1.8-2.1 range was used for the c DNA synthesis.

2.2.18.4 c DNA Synthesis

The reagents provided with the c DNA synthesis kit (Bio Rad) were added to the RNA template in variable amounts making a final volume up to 20µl. The complete reaction mix was incubated in the thermal cycler using the following protocol: priming for 5min at 25^0C, reverse transcription for 20 min at 46^0C, RT inactivation for 1min at 95^0C and holding at 4^0C.

2.2.18.5 Setting up the reaction

In a PCR reaction plate, template c DNA, forward primer, reverse primer, SYBR® Green master mix and nuclease free water were added in each reaction well, forming the final volume of 10µl. The plate was sealed and placed in micro centrifuge for the contents to evenly mix. The reactions were run in the real time PCR machine (CFX96 ^{TM} Real-Time System, Bio-Rad, UK).

2.2.18.6 Statistical analysis

All the data were analysed using CFX software to determine the gene expression levels and normalised against the internal gene, GAPDH according to Livak and Schmittgen, 2001 using delta CT methods. Each experiment was repeated 3 times in 3 individuals and values are presented as mean and standard deviation (SD). The differences between various treatment groups were determined by performing normality checks. Data were analysed using One-way ANOVA and t-test on Graph Pad Prism 7.02.

2.2.19 Immunocytochemistry (ICC)/Immunofluorescence (IF)

2.2.19.1 Cell Culture

Isolated lymphocytes were counted and re-suspended in complete media (RPMI 1640 with 25mM HEPES and L-Glutamine, 1% penicillin-streptomycin and 15% foetal bovine serum) and treated with chemicals for 24 hrs. The next day the cells were pelleted and washed with cold PBS twice followed by re-suspension in 100µl PBS and dropped on clean glass slides.

2.2.19.2 Fixation

Dried slides were fixed in 4% formaldehyde for 15 mins at RT. Then cells were washed with cold PBS 3 times for 5 mins each.

2.2.19.3 Permeabilisation

The lymphocytes were incubated in permeabilisation buffer, PBST (0.1% Triton X-100 in PBS) for 10mins at RT followed by washing steps with PBS.

2.2.19.4 Blocking

Unspecific sites were blocked in blocking buffer containing 5% BSA in TBS (0.1% Tween 20 in PBS) for one hour at RT followed by the washing steps.

2.2.19.5 Immunodetection

Cells were incubated with primary antibody overnight at 4^0C and then washed with PBS 3 times for 5 mins each. Secondary antibody incubation was carried out in the dark at RT for one hour followed by the washing steps.

2.2.19.6 Immunostaining

Slides were stained with Dapi (5mg/ml) for 1 min and rinsed with PBS. Gold antifade reagent was used as a slide mountant and coverslips were placed on top and allowed to dry in the dark.

2.2.19.7 Statistical Analysis

Duplicate slides were produced for each treatment group. 100 cells per slide were counted at 100X magnification (with oil immersion) using a fluorescence microscope with fitted CCD camera (Nikon Digital Sight DS-SMC, Surrey, UK)

2.2.20 Analysis of confounding factors

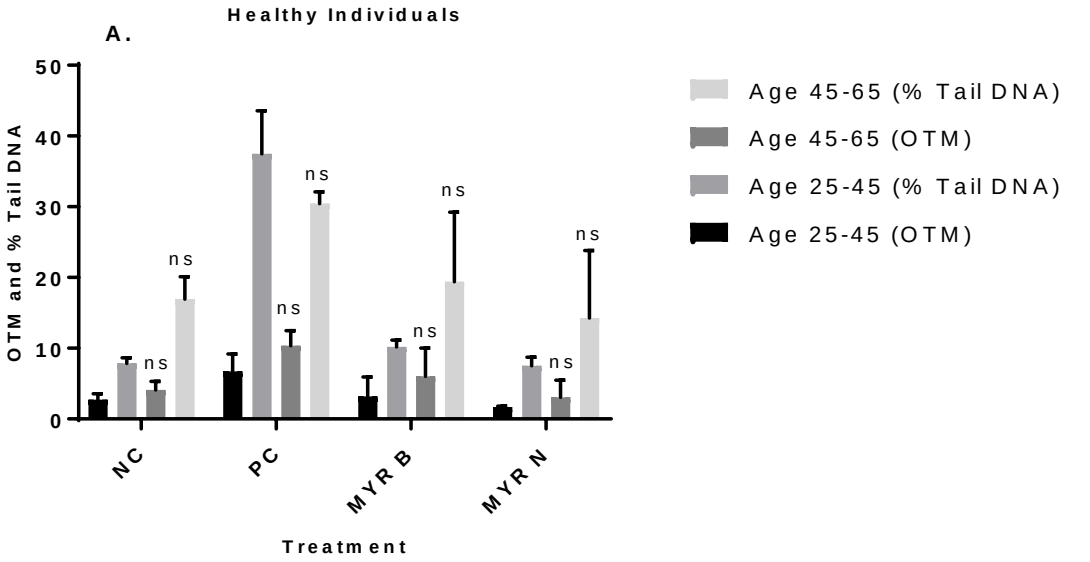
Confounding factors such as age, gender, ethnicity, lifestyle (diet, smoking and drinking habits) and environmental factors are very important and need to be considered when assessing the DNA damage in humans because these may contribute towards the damage and therefore could induce genotoxicity (Fenech, 2007). Lifestyle variables such as smoking and alcohol consumption may render individuals susceptible to cancer and other diseases because they are known carcinogens. Age could also contribute towards the DNA damage due to compromised immune system and DNA repair mechanism. Therefore, we determined the effects of these variables on comet values in both the healthy individuals and pre-cancerous patients. In the present study, we found no relationship among the confounding factors regardless of the treatment groups and these do not seem to be contributing much towards DNA damage as shown in results from healthy individuals which have little basal damage. All patients were suffering from various diseases which were the main cause of a high level of damage in the patient groups.

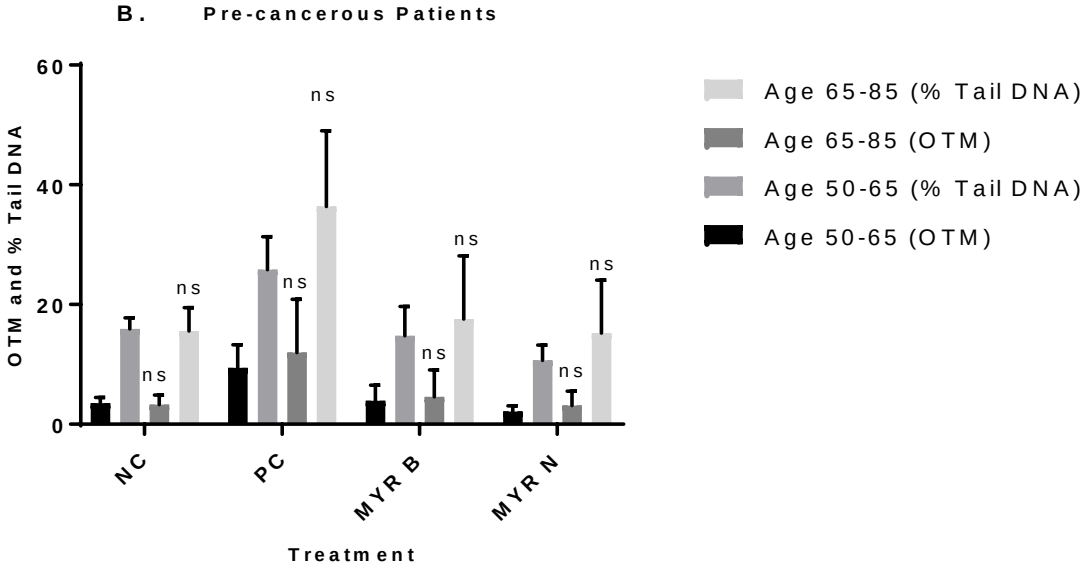
Myricetin in both forms has not shown any significant genotoxicity. Moreover, MYR N has shown protective effects in patient groups but the values are not significant.

2.2.20.1 Age

Age varies greatly between both the groups the healthy individuals and pre-cancerous patients. Most of the healthy individuals were under 50 years of age whereas all the patients were above 55 years old. There could be some contribution due to age in the patient group. However, these were the patients available at the time of study and it was not possible to match them better to the

controls. The effect of age was determined in healthy individuals, pre-cancerous patients and MM patients (Figure 2.1). Our data from the Comet assay show no significant difference on DNA damage in any of the age group.





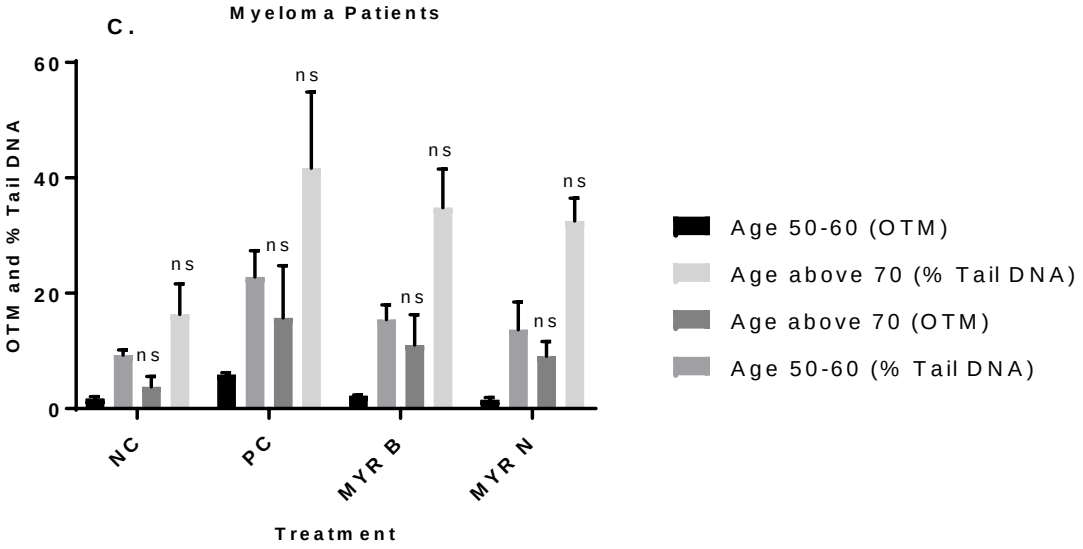


Figure 2.1 Shows the means of % tail DNA and Olive tail moment OTM in the peripheral blood lymphocytes of different age groups in (A) healthy individuals, (B) pre-cancerous and (C) MM patients in the Comet assay after different treatments with the PC (H_2O_2)(50μM), MYR B (10μM) and MYR N (20μM) as well as the negative control of untreated lymphocytes (NC). Bars indicate standard errors (SEs). Not significant: ns. Statistics show the comparison between the OTM of one investigative group with OTM of other and between % tail DNA of one group with % tail DNA of other.

2.2.20.2 Gender

There were no significant differences observed between the groups in lymphocytes of healthy (figure 2.2a) neither patient (figure 2.2b) males nor females when treated with various treatments.

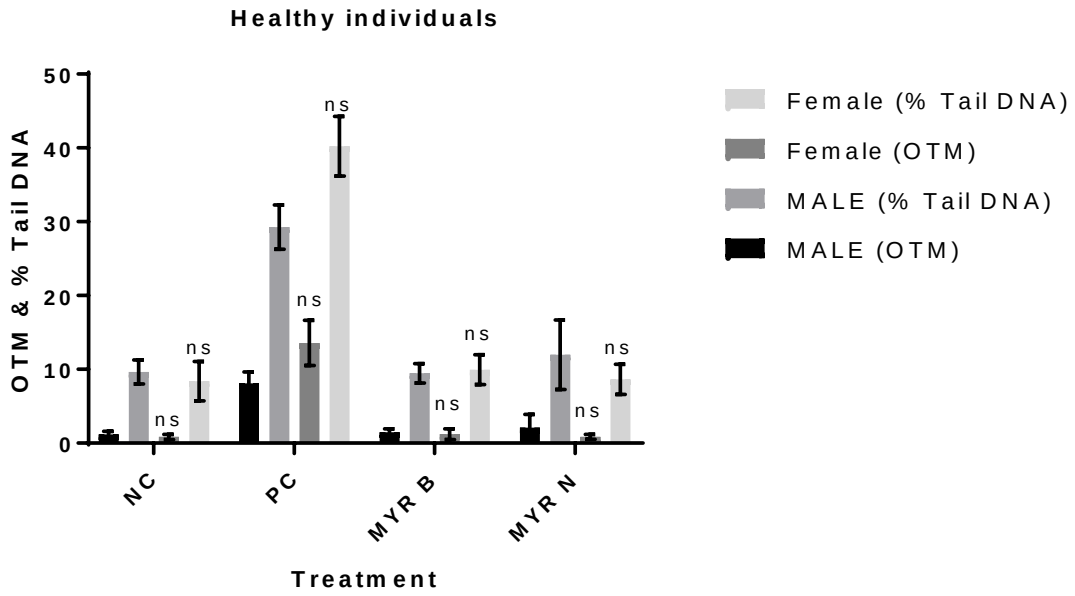


Figure 2.2a Histogram showing the means of Olive tail moment and % tail DNA in the peripheral blood lymphocytes of males and females healthy individuals in the Comet assay after different treatments with the PC (H_2O_2)(50µM), MYR B (10µM) and MYR N (20µM) as well as the negative control of untreated lymphocytes (NC). Bars indicate standard errors (SEs). Not significant: ns, Statistics show the comparison between the OTM of one investigative group with OTM of other and between % tail DNA of one group with % tail DNA of other.

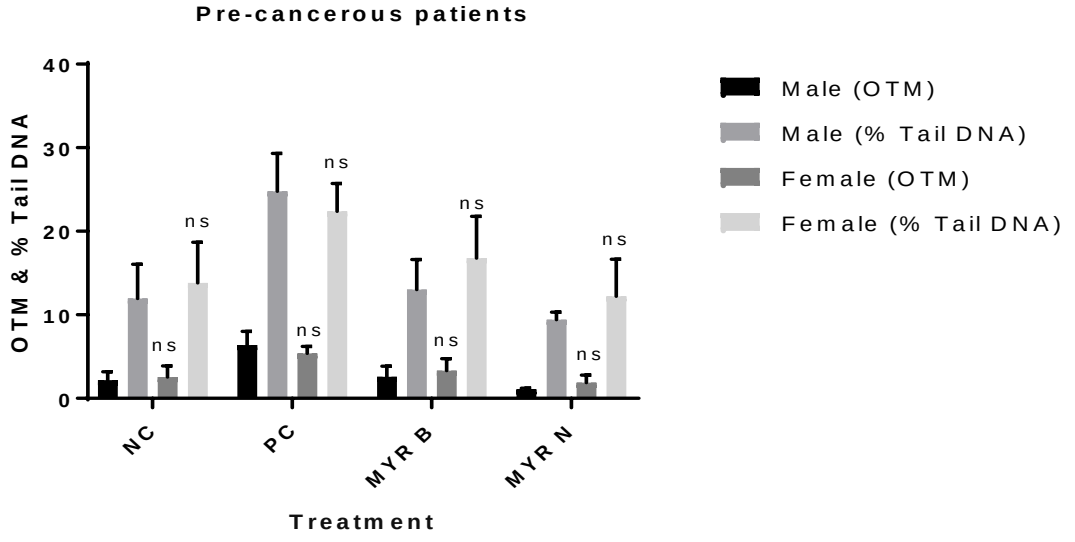


Figure 2.2b Histogram showing the means of Olive tail moment and % tail DNA in the peripheral blood lymphocytes of males and females pre-cancerous patients in the Comet assay after different treatments with the PC (H_2O_2)(50µM), MYR B (10µM) and MYR N (20µM) as well as the negative control of untreated lymphocytes (NC) for 30 minutes. Bars indicate standard errors. ns: Not significant, Statistics show the comparison between the OTM of one investigative group with OTM of other and between % tail DNA of one group with % tail DNA of other.

2.2.20.3 Smoking habits

The healthy (figure 2.3a) and patient (figure 2.3b) smoker groups have shown more DNA damage at basal levels when compared to respective non-smoker groups but the value are not statistically significant. The PC control has induced significant levels of DNA damage; however MYR B and MYR N have not shown any differences regardless of group types.

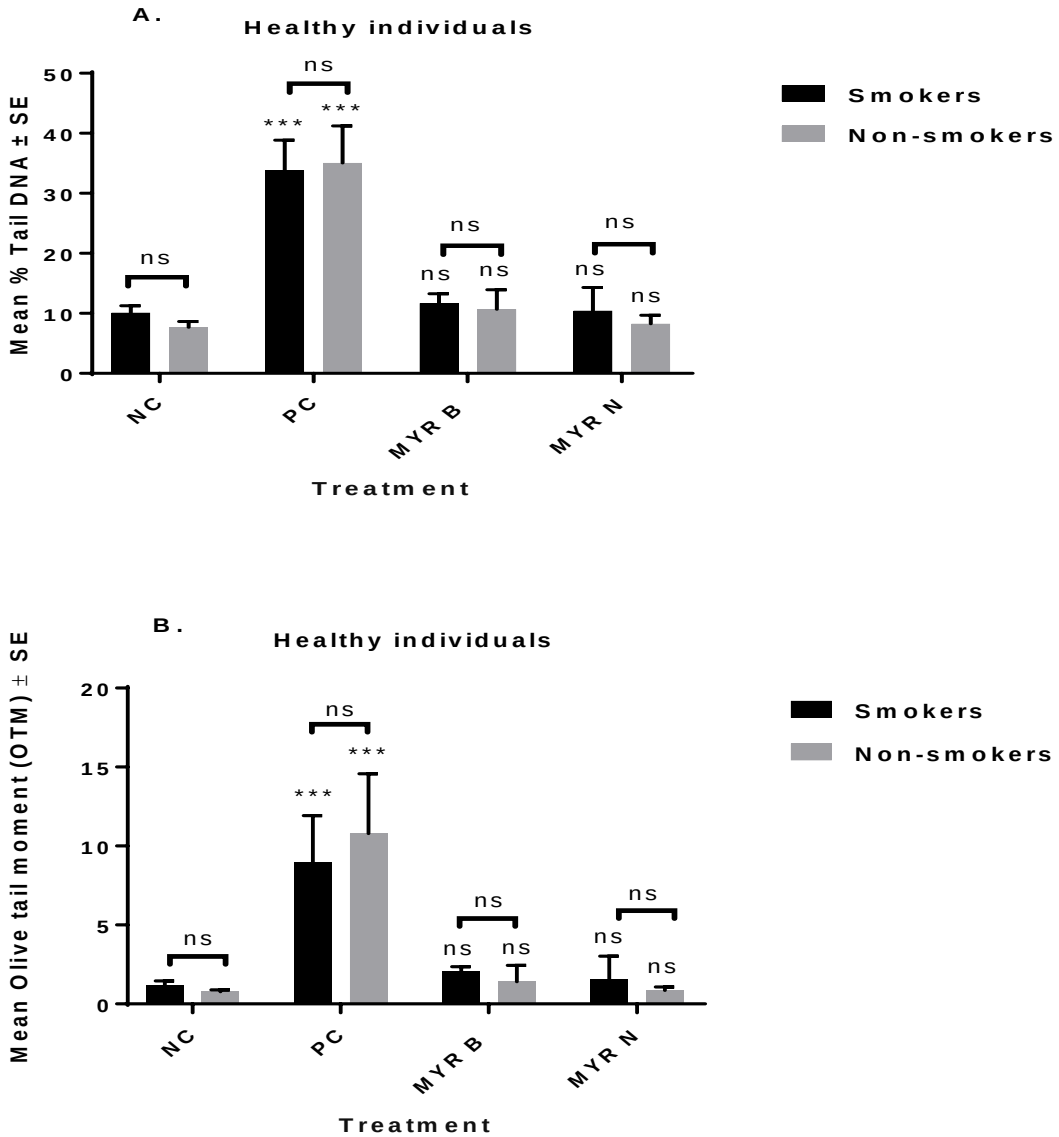


Figure 2.3a Shows the means of % tail DNA (A) and Olive tail moment OTM (B) in the peripheral blood lymphocytes of smokers and non-smokers healthy individuals in the Comet assay after different treatments with the PC (H_2O_2)(50μM), MYR B (10μM) and MYR N (20μM) as well as the negative control of untreated lymphocytes (NC). Bars indicate standard errors (SEs) and the horizontal lines indicate the differences between the groups. Not significant: ns, *** $p < 0.001$. Statistics under the horizontal lines show comparison with NC.

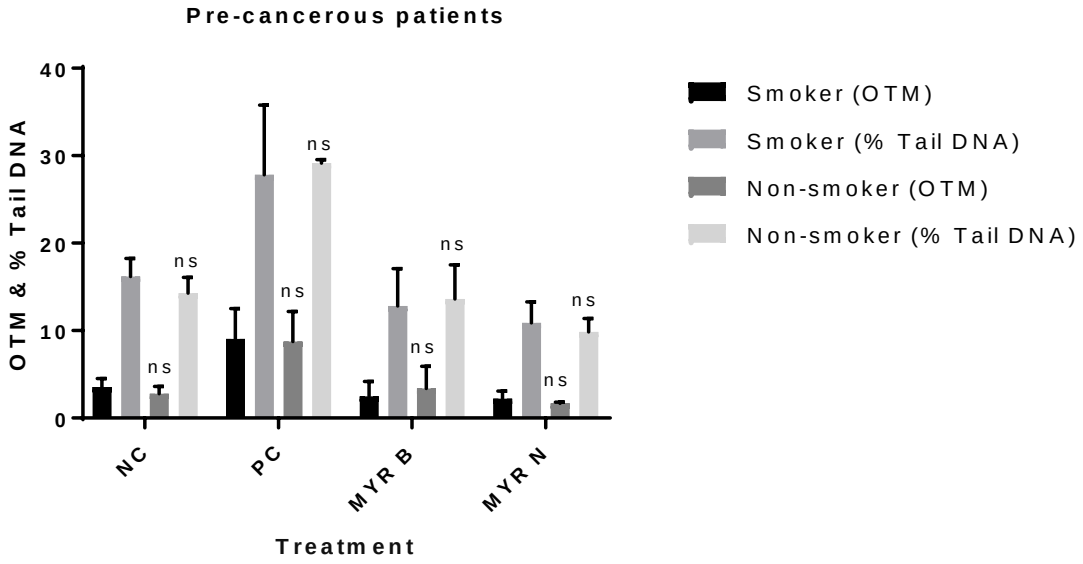


Figure 2.3b Shows the means of % tail DNA and Olive tail moment OTM in the peripheral blood lymphocytes of smokers and non-smokers pre-cancerous patients in the Comet assay after different treatments with the PC (H_2O_2)(50μM), MYR B (10μM) and MYR N (20μM) as well as the negative control of untreated lymphocytes (NC). Bars indicate standard errors (SEs). Not significant: ns. Statistics show the comparison between the OTM of one investigative group with OTM of other and between % tail DNA of one group with % tail DNA of other.

2.2.20.4 Ethnicity

The figure below demonstrates no statistically significant differences between the healthy (Figure 2.4a) and patient (figure 2.4b) different ethnic groups.

Asian patients showed more basal DNA damage than Caucasians but the values were not significant.

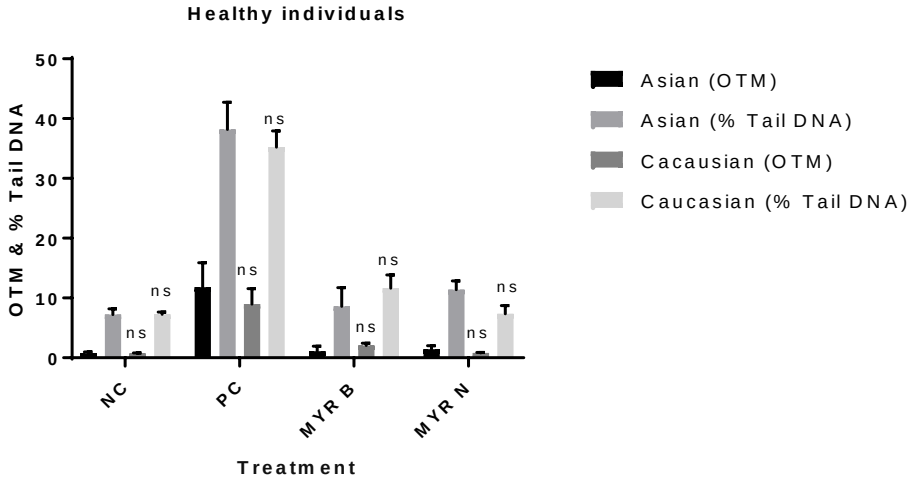


Figure 2.4a Histogram showing the means of Olive tail moment and % tail DNA in the peripheral blood lymphocytes of Asian and Caucasian healthy individuals in the Comet assay after different treatments with the PC (H_2O_2)(50μM), MYR B (10μM) and MYR N (20μM) as well as the negative control of untreated lymphocytes (NC) for 30 minutes. Bars indicate standard errors. Not significant: ns. Statistics show the comparison between the OTM of one investigative group with OTM of other and between % tail DNA of one group with % tail DNA of other.

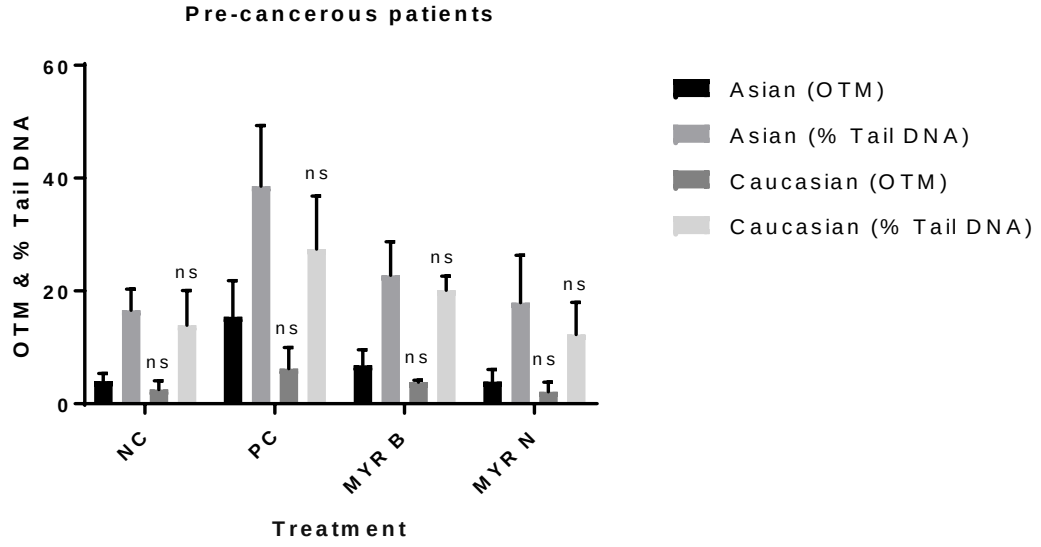


Figure 2.4b Histogram showing the means of Olive tail moment and % tail DNA in the peripheral blood lymphocytes of Asian and Caucasian pre-cancerous patients in the Comet assay after different treatments with the PC (H_2O_2)(50μM), MYR B (10μM) and MYR N (20μM) as well as the negative control of untreated lymphocytes (NC) for 30 minutes. Bars indicate standard errors. ns: Not significant, Statistics show the comparison between the OTM of one investigative group with OTM of other and between % tail DNA of one group with % tail DNA of other.

Chapter 3: Protective effects of myricetin bulk and nanoparticle forms on peripheral lymphocytes from pre-cancerous patients compared to those from healthy individuals

3.1 Introduction

Myricetin belongs to the family flavonoids; flavonols, with various antioxidant properties. The three OH-groups in ring B (3′,4′,5′-position) make it a stronger antioxidant by scavenging ROS through oxidation of these hydroxyl groups. Myricetin is usually contributed by vegetables, berries, tea, fruits, nuts and red wine to our diet (Ross and Kasum, 2002). It is mainly found in plant derived foods especially tea, cranberries and grapes (Büchter et al., 2013). Its average intake varies, subject to the diet consumed and lifestyle factors. Myricetin is made from parental compound taxifolin through an intermediate, dihydromyricetin and can also be directly produced from kaempferal (Flamini et al., 2013). Structurally it is very similar to quercetin bearing dihydroxyl groups at position 3' and 4'. It has an extra hydroxyl group at position 5' which makes it an even better antioxidant. H atom from 4' is easily abstracted by radical species forming a flavonoid radical and remaining O^- radical is efficiently stabilised due to neighbouring hydroxyl groups on both sides. Myricetin exhibits many health beneficiary effects including, antioxidant, anti-photo aging, anti-cancer, anti-hypertension, immunomodulatory, anti-inflammatory, anti-allergic, analgesic, antimicrobial, anti-diabetic and protective against cardiovascular diseases (Semwal, 2016).

Maintaining homeostasis between the damage and repair mechanisms is crucial for our genome integrity. For this a eukaryotic cell bears a highly specialised system called 'cell cycle checkpoints' that keeps check on every step of the cell cycle and guarantees a healthy and smooth progression (Ciccia and Elledge, 2010; Hartwell and Weinert, 1989). The tumour suppressor protein, P53, is the key regulator of the cell cycle and is considered as the main

mediator of checkpoint triggered arrest in G1 phase and its function is critical for defence against cancer development. Various cellular stress signals including hypoxia, DNA damage, nucleotide deprivation, different infections and oxidative stress etc. lead to the initiation of P53 as a transcription factor (Cox and Lane, 1995). Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related kinase (ATR) are phosphatidylinositol-3-like kinases crucial for detecting the DNA damage and initiating the cell cycle checkpoints, repair and apoptosis (Sarkaria et al., 1999). ATM kinase is considered as an important mediator of H2AX phosphorylation in reaction to double strand breaks (DSB) formation (Bakkenist and Kastan, 2003). The main pathway which regulates the ATM expression is through DSBs but can possibly be triggered in all cell cycle stages (Savitsky et al., 1995). Following DNA damage, ATM is required to efficiently stimulate the cell cycle checkpoints and homologous recombination including checkpoints protein P53 and checkpoint kinase 2 (Chk2) (Kastan and Lim, 2000). ATM has previously been shown triggering, P53-induced cell cycle arrest following DNA damage (Kang et al., 2005). ATM and ATR both directly stabilize p53 on Ser15 and Ser37 respectively, by phosphorylation *in vivo* (Siliciano et al., 1997). Germline ATM mutations can result in increased sensitivity to ionising radiation, a predisposition to cancer and immunodeficiency (Barzilai and Yamamoto, 2004). Upon activation, ATM goes through dimer separation and auto-phosphorylation at Ser 1981 (Bakkenist and Kastan, 2003).

Although, past studies have successfully demonstrated characteristics of myricetin against different diseases (Semwal, 2016), this is the first study to our knowledge that the effects of the particle size of myricetin have been investigated at the cellular level *ex vivo/in vitro*. In the present study, DNA damage in peripheral lymphocytes of pre- cancerous patients and healthy

individuals have been compared after treatment with myricetin nanoparticle (MYR N) and bulk (MYR B) forms in the Comet and micronucleus assays. Effects of MYR B and MYR N on protein expression of the tumour suppressor gene, P53 were investigated using Western blotting. Their effects on gene expression of ATM and P53 were analysed at m RNA level using the real-time PCR technique.

3.2 Materials and methods

The blood samples from healthy individuals and pre-cancerous patients used in this experimental chapter are listed below in table 3.1 and 3.2, respectively.

No	Age	Ethnicity	Gender	Smoking history	Family history
1	48	CAUCASIAN	M	NO	NONE
2	28	CAUCASIAN	M	NO	NONE
3	27	AFRICAN	M	YES	NONE
4	38	CAUCASIAN	M	NO	NONE
5	60	CAUCASIAN	F	NO	NONE
6	40	ARAB	M	NO	NONE
7	45	CAUCASIAN	M	YES	NONE
8	55	CAUCASIAN	M	NO	NONE
9	35	CAUCASIAN	M	YES	NONE
10	25	CAUCASIAN	M	NO	NONE
11	44	ASIAN	M	YES	NONE
12	28	CAUCASIAN	M	NO	NONE
13	23	CAUCASIAN	F	NO	NONE
14	27	CAUCASIAN	M	NO	KIDNEY CANCER
15	33	ARAB	M	YES	NONE
16	47	ASIAN	M	YES	NONE
17	28	CAUCASIAN	M	NO	NONE
18	42	ASIAN	M	NO	NONE
19	48	ASIAN	M	NO	NONE
20	60	ASIAN	M	YES	NONE

Table 3.1 Healthy blood samples used in this chapter

No	Age	Ethnicity	Gender	Smoking history	Family history	Medical condition
1	79	CAUCASIAN	M	NO	NONE	MGUS, COPD, MONOCLONAL B CELL LYMPHOCYTOSIS
2	80	CAUCASIAN	F	NO	NONE-	MGUS
3	78	CAUCASIAN	M	NO	NONE-	MGUS
4	56	CAUCASIAN	F	YES	LEUKAEMIA & BRAIN TUMOUR	MGUS
5	77	CAUCASIAN	M	NO	NONE	MGUS
6	75	CAUCASIAN	M	NO	NONE	MGUS
7	80	CAUCASIAN	M	NO	CANCER POSITIVE	MGUS
8	81	CAUCASIAN	F	NO	BOWEL& STOMACH	MGUS
9	63	CAUCASIAN	M	YES	NONE	MGUS
10	55	CAUCASIAN	M	NO	NONE	MGUS
11	83	CAUCASIAN	M	NO	NONE	MGUS, BENIGN PROSTATIC HYPERPLASIA
12	63	CAUCASIAN	M	YES	NONE	MGUS
13	74	CAUCASIAN	M	NO	NONE	MGUS COPD
14	63	CAUCASIAN	F	YES	ARTHRITIS	MGUS, COPD
15	66	CAUCASIAN	F	NO	BREAST CANCER	MGUS
16	52	CAUCASIAN	M	YES	NONE	MGUS
17	83	CAUCASIAN	M	NO	NONE	MGUS
18	60	ASIAN	F	NO	NONE	MGUS
19	75	CAUCASIAN	F	NO	MASTECTOMY	MGUS
20	69	CAUCASIAN	M	NO	STOMACH AND LUNG	MGUS

Table 3.2 Brief information about pre-cancerous patients samples used in this chapter

3.2.1 Preparation of myricetin bulk and nanoparticle forms, their concentration determination and zeta potential of nanoparticles

Myricetin was purchased from Fisher Scientific, UK, as a powder (>96% purity). Suspensions of myricetin bulk and nano form were made in an excipient mixture (containing 7% (w/w) solid loads of myricetin in a different medium comprising of hydroxypropyl methylcellulose (HPMC) (0.5 % w/w), sodium lauryl sulphate (SLS) (0.1% w/w), ethanol (0.8% w/w), polyvinylpyrrolidone (PVP) K-30 (0.5% w/w) and purified water). Dose–response experiments were used to determine the optimal doses which were used throughout the study. These were 10µM for myricetin bulk and 20µM for myricetin nano form. H_2O_2 (50µM) was used throughout the study as the positive control. The mean particle sizes of

myricetin nano in the stock solutions were measured using a Zetasizer Nano ZS-90 Model ZEN3600 (Malvern Instruments Ltd, UK) by Photon Correlation Spectroscopy.

3.2.2 Transmission electron microscopy (TEM) Analysis

To visualise the individual particles and their shapes, transmission electron microscopy TEM Tecnai 12, (FEI Company, Netherlands) was used. Please refer to 2.2.6 from chapter 2 for the full procedure.

3.2.3 Isolation of lymphocytes and Cell viability measurement

Isolated lymphocytes were treated with chemicals for 1, 24 and 48 hrs and subjected to trypan blue dye exclusion to measure all membrane viability and integrity. Please refer to (chapter 2) 2.2.9 and 2.2.11 for comprehensive procedures followed.

3.2.4 Cell survival rate and cytotoxicity determination

The cytotoxicity of chemicals was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance. Please refer to 2.2.12 (chapter 2) for details.

3.2.5 DNA damage determination using the Comet assay

The patient blood samples were kindly provided by the department of haematology, Bradford Royal Infirmary (BRI), Bradford, UK. The healthy blood samples were collected from healthy volunteers. The blood was diluted (1:1) with RPMI medium (Invitrogen, UK) and supplemented with 10% DMSO and

instantly stored at -80^{0}C. Please refer to 2.2.13 (chapter 2) for the assay procedure.

3.2.6 Micronucleus (MN) assay

Lymphocytes from patients and healthy individuals were treated with 10 and 20µM of MYR B and MYR N respectively to determine the frequency of MNi, which was used as a marker of DNA damage. The drug concentrations used for the assay were optimum doses, chosen from dose response experiments which induced no cytotoxicity. Please refer to 2.2.14 (chapter 2) for the assay procedure.

3.2.7 Western blot analysis

Lymphocytes were seeded in 6-well plates at concentration of 10^6cells/well, incubated overnight and treated with chemicals (MYR B 10µM and MYR N 20µM) for 24hrs. Refer to 2.2.15 (chapter 2) for detailed technique.

3.2.8 Real-time RT-PCR analysis

Isolated lymphocytes were seeded in 6-well plates and treated with chemicals for 24 hrs. Please refer to 2.2.18 (chapter 2) for details.The primers used are listed in table 3.3 below.

Genes	**Primer Sequence 5'-3'**
ATM	ACCATTGTAGAGGTCCTTC GTCTCATTAAGACACGTTCAG
P53	CTCCTCAGCATCTTATCCGAGT GCTGTTCCGTCCCAGTAGATTA
GAPDH	TGCACCACCAACTGCTTAG AGTAGAGGCAGGGATGATGTTC

Table 3.3 Primers used for Real-Time PCR analysis

3.2.9 Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). Graph Pad prism was used to perform statistical calculation. The results were analysed using t-tests, one-way analysis of variance (ANOVA) and two-way ANOVA to test differences between each treatment and control. A p-value of <0.05 was considered statistically significant.

3.3 Results

3.3.1 Particle size determination of Myricetin bulk and nanoparticles

Although, the particle size determined for myricetin NPs was >100nm, we still refer to them as NPs of myricetin. Nano particles of myricetin suspended in excipient mixture were compared to the solution prepared from its bulk powder in the same excipient mixture. Results show the average particle of myricetin bulk and nanoparticles as 1737 and 161nm, respectively (table 3.4 and 3.5). Nanoparticles of myricetin were much smaller compared to the bulk particles. Although both preparations were at a concentration below the solubility of myricetin in the final cell culture media, it has been shown none the less that nanoparticles do provide higher bioactivity and enhanced dissolution rate than the bulk form. This is because upon addition to the cell culture media, nanoparticles are capable of providing higher concentration of the drug in the immediate vicinity (or inside) the cells. Nanoparticles have been shown to penetrate cell membranes via phagocytosis and endocytosis and thus provide a higher effective dose within the cells (Gradinaru et al., 2010). It is reported that particles in the size range of 250 nm to 3μm are mainly internalised by phagocytosis whereas those below 200nm are mainly up taken by endocytosis

(Amit et al., 2014). Nano medicine has been shown to exhibit different biological activity compared to solutions of the same drug.

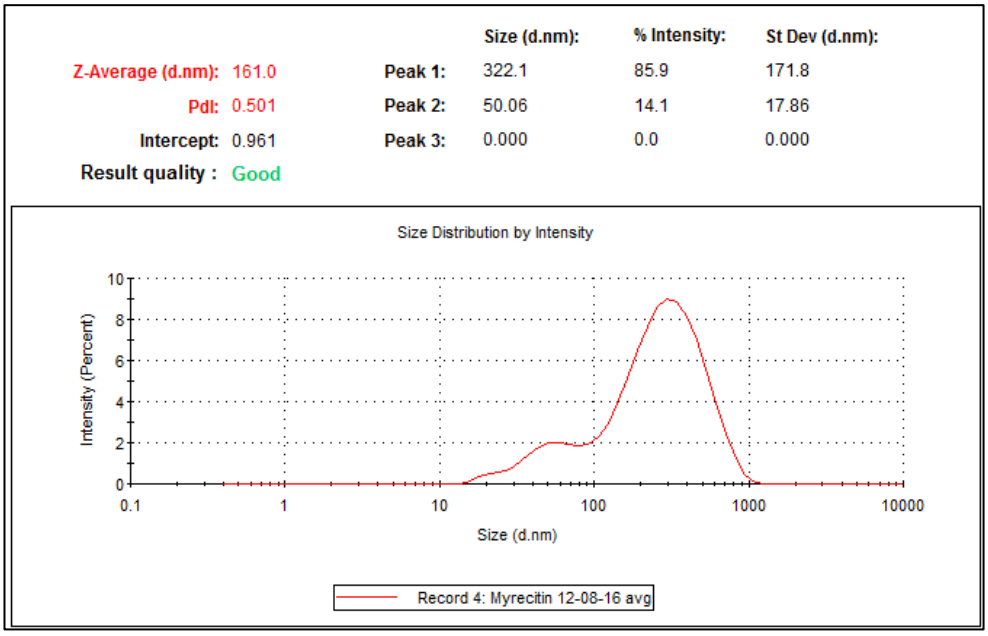


Table 3.4 The peak obtained for myricetin NPs during the procedure

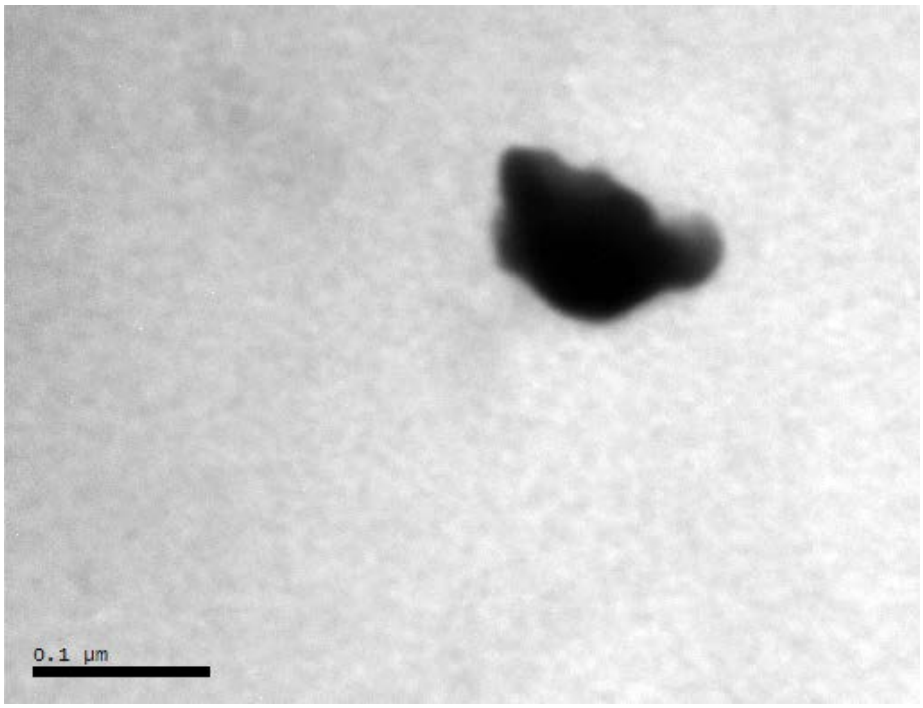
Suspension name	Zeta average particle size (nm)	Polydispersity Index	Intercept	Quality
Myricetin nano	161.0	0.501	0.961	Good
Myricetin bulk	1737	-	-	Good

Table 3.5 Average particle size of Myricetin bulk and NPs

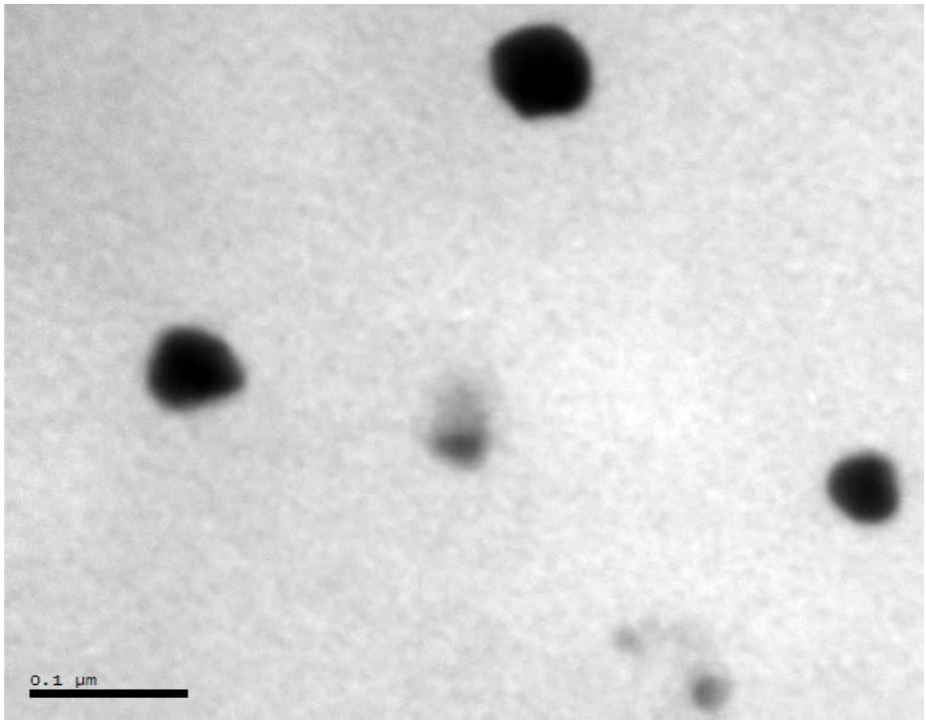
3.3.2 TEM analysis of Myricetin bulk and nanoparticles

TEM analysis was used to visualise myricetin bulk and NPs at different magnifications ranging from 10-150k. The scale bar at the bottom left of each image below gives the approximate length of respective particle shown in that image. To reduce and avoid the possibility of dust particles' clinging to the surface of charged TEM grids, sample preparation was carried out in a clean fume hood cabinet using covered petri dishes and other equipment. Hence, chances of dust particles overlapping with myricetin particles were made limited, enhancing the effectiveness of TEM visualization of myricetin bulk and

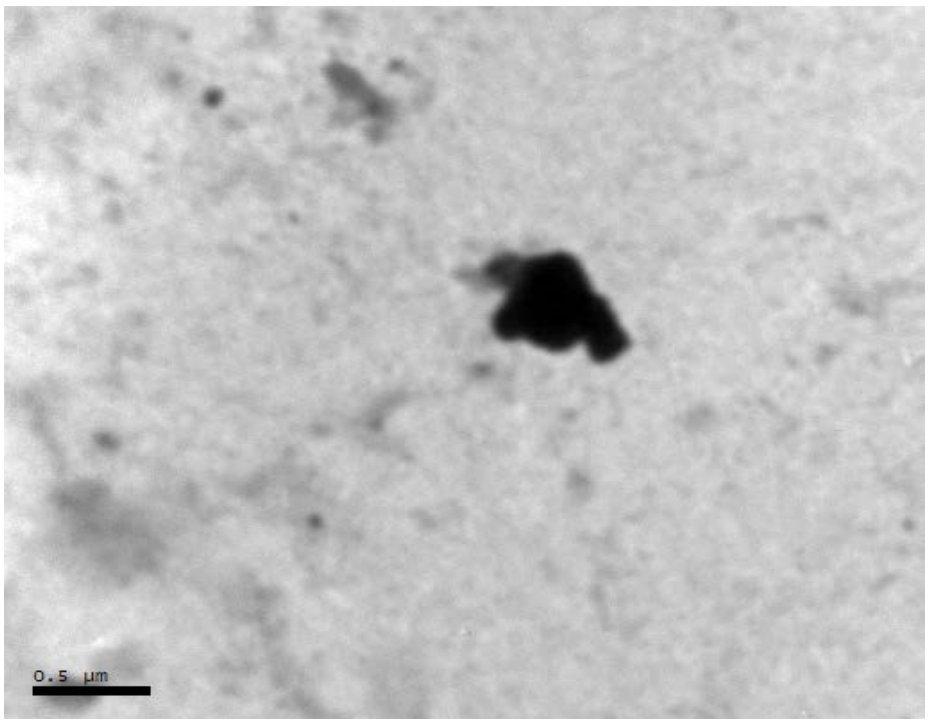
NPs. Results have revealed an irregular shaped aggregate of one or 2 NPs of myricetin shown in figure 3.1 A and B. However, myricetin bulk particles are more irregular in shape (single particle shown in figure 3.1 C) and found to form colonies by sticking with each other shown in figure 3.1 D.



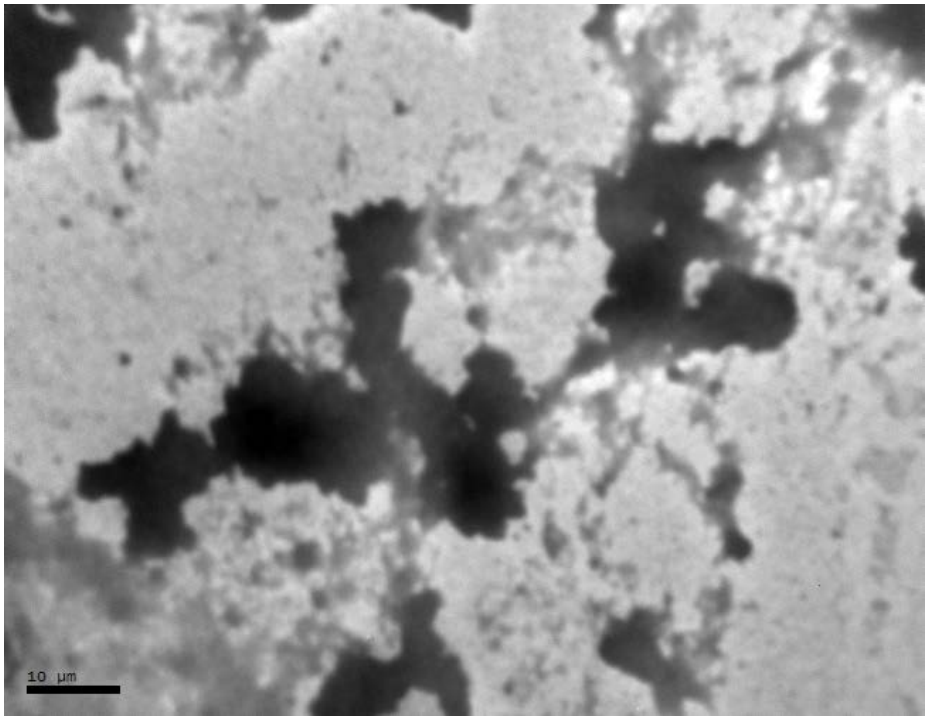
A. Myricetin NP (150 k magnification)



B. Myricetin NPs cluster (150 k magnification)



C. Myricetin bulk particle (20 k magnification)



D. Myricetin bulk cluster (10 k magnification)

Figure 3.1 (A-D) The transmission electron microscopy (TEM) of MYR B and MYR N particles

3.3.3 Concentration dependent responses of myricetin bulk (MYR B) and myricetin nano (MYR N) to determine genotoxicity

Genotoxicity was assessed using a range of concentrations (10μM to 80μM) of MYR B and MYR N. Results shown below using two parameters of the Comet assay, % tail DNA and OTM have demonstrated that myricetin in both forms has caused significant dose dependent genotoxicity in lymphocytes at higher concentrations except for MYR B 10μM and MYR N at 20μM, which did not induced any significant levels of genotoxicity determined by both parameters. Although % tail DNA data shows that MYR N (10μM) and MYR B (20μM) also caused non-significant levels of genotoxicity but in current study, we only used the optimal concentrations for MYR B and MYR N, 10μM and 20μM respectively which have induced no significant genotoxicity confirmed by both parameters of the Comet assay. Flavonoid compounds have this ability to cause adverse effects at slightly different concentrations as evaluated in figure 3.2 where MYR N 20μM has been shown safer than MYR N 10μM, therefore; we used the safer concentrations with no significant genotoxicity. As we are conducting an *in vitro* study so these chosen concentrations are safe and acceptable to use on lymphocytes knowing that the *in vivo* physiological concentration range of myricetin is from 5-10μM (Peng and Kuo, 2003). Compilation of various studies presented by Semwal (2016) has shown wide concentration ranges used for myricetin which are well above its physiological range and produced protective effects against different cancers and cardiovascular diseases.

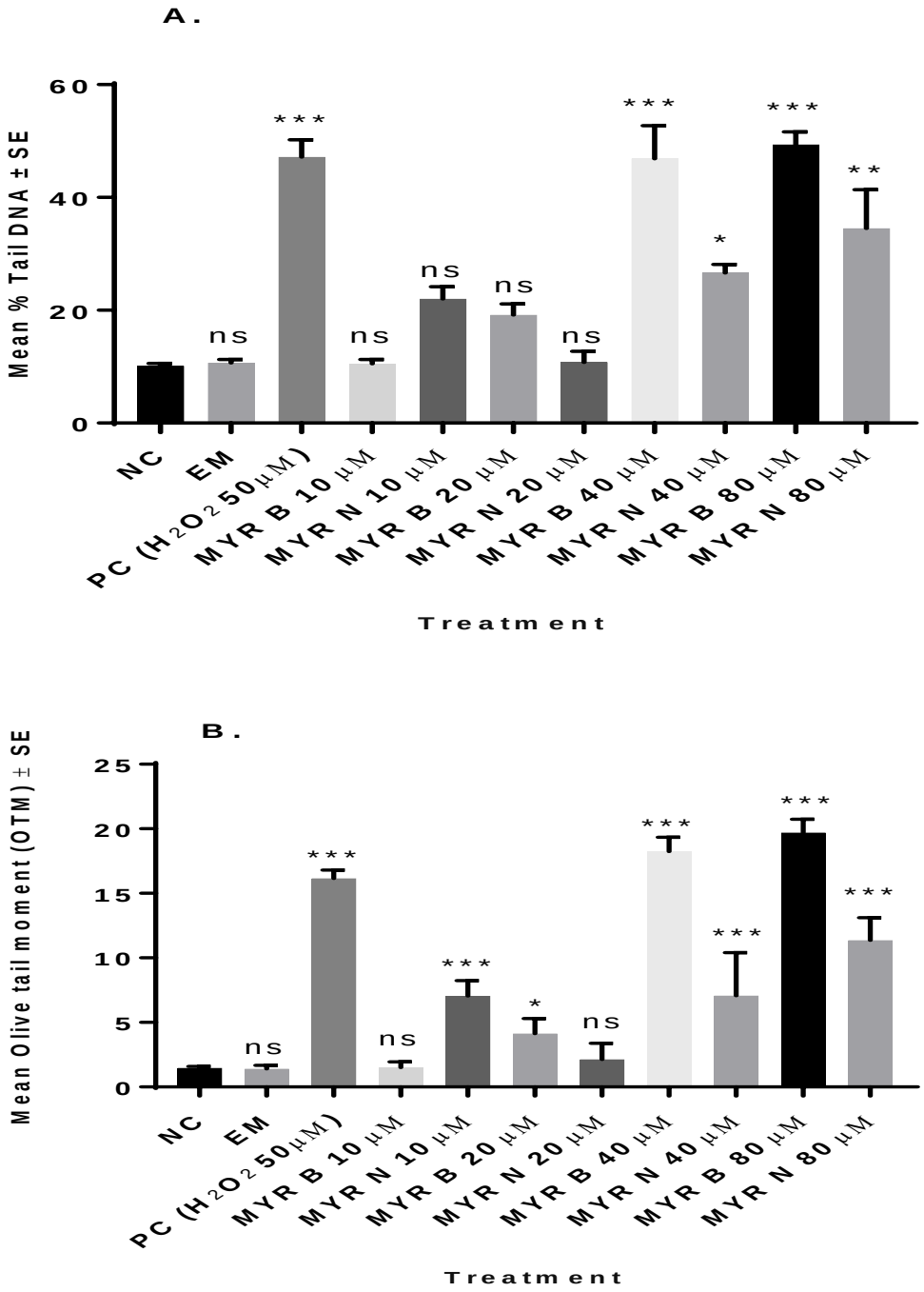


Figure 3.2 Concentration dependent responses of MYR B and MYR N in healthy lymphocytes showing mean % tail DNA (A) and OTM (B). All data have been expressed as mean ± standard errors (SE) in 20 healthy individuals, measuring 100 cells each. *In vitro* treatment of healthy lymphocytes with different concentrations of MYR B and MYR N in the Comet assay has shown that MYR B (10μM) and MYR N (20μM) produced non-significant levels of the DNA damage which was comparative with the negative control (NC). Therefore these optimum concentrations have been used throughout the study. Other treatment groups included, excipient mixture (EM) (0.1%) and PC (H_2O_2 50μM). The stars indicate significant difference between NC and various treatment groups ($P<0.05$ significantly different), ns indicates not significant, $^{***}P<0.001$, $^{**}P=0.006$, $^{*}P<0.01$

3.3.4 Cytotoxicity of Myricetin bulk and nanoparticles in lymphocytes from healthy vs patient groups

This assay can be used to determine the cytotoxicity of different chemical agents. The MTT dye, a yellow tetrazol is reduced to purple formazan by the NAD(P)H-dependent cellular oxidoreductase enzymes under certain conditions, in the living cells. Then DMSO is added to dissolve the formazan crystal in the solution and absorbance of this coloured solution can be measured at a certain wavelength, usually 560nm, using a spectrophotometer. The results have revealed a significant ($P<0.01$, $P<0.003$) time and concentration-dependent cytotoxicity which was measured at 570nm by optical density after exposure to different treatment groups i.e. MYR B (10-40μM), MYR N (10-40μM), PC (50μM) and EM (0.1%) for various time periods. Optical density represents the mean ± SE of triplicate MTT assays on isolated lymphocytes cells from patients and healthy individuals treated with the above mentioned chemicals as well as the untreated group. Fig 3.3 shows the effects of various treatment groups on the viability of healthy and pre-cancerous patients' lymphocytes at different times (1, 24 and 48hrs) compared to the respective untreated group where H_2O_2 significantly reduced the viability after 48hrs. MYR B and MYR N also exhibited a time and concentration dependent decrease in cell viability. MYR B (40μM) and MYR N (40μM) reduced the viability in lymphocytes to 88% and 86% from healthy individuals and to 86% and 83% from pre-cancerous patients respectively, after a 48 hours treatment. Neither of the concentrations of MYR B and MYR N reduced viability less than 80% in either group.

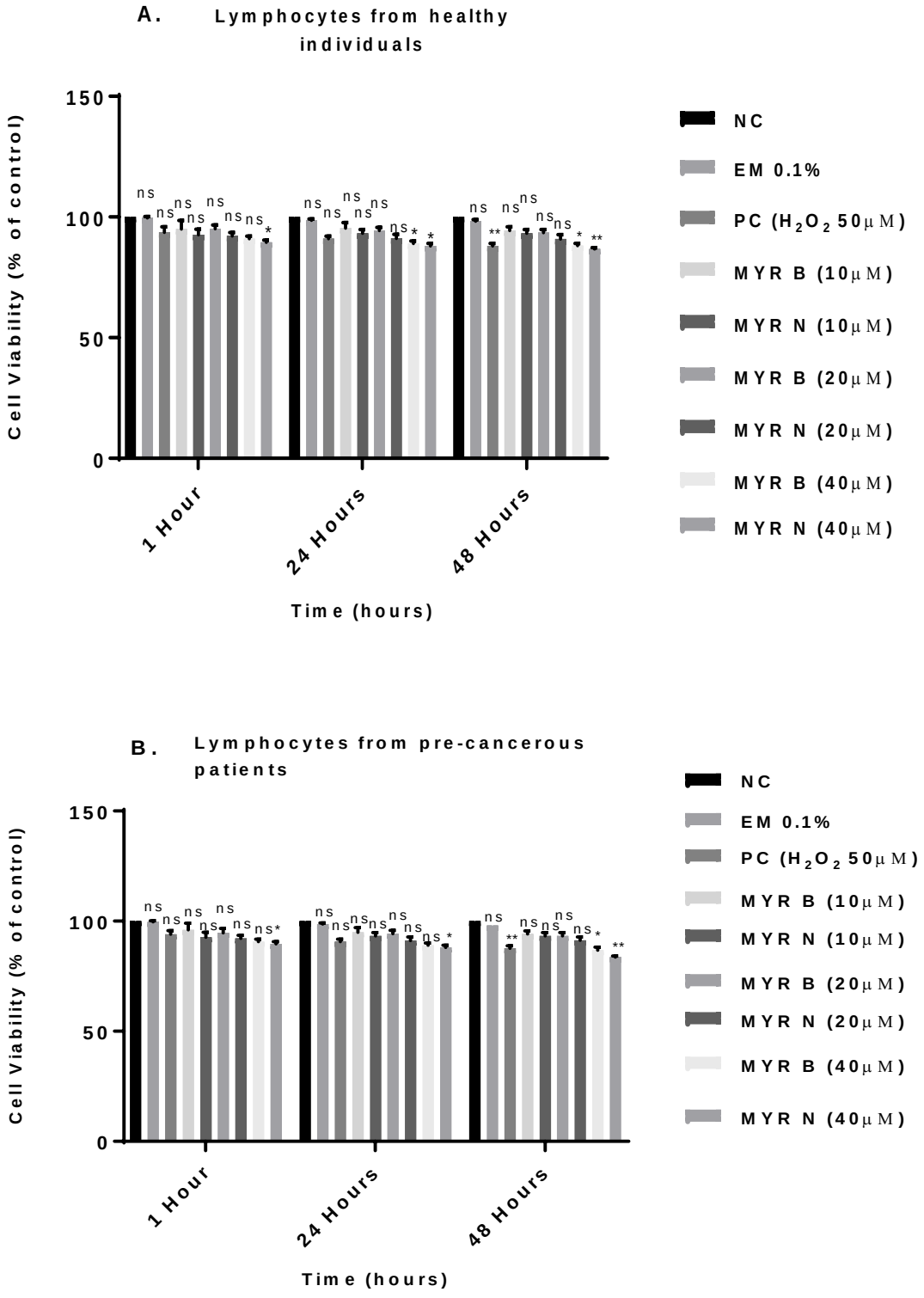


Figure 3.3 Shows the % cell viability of lymphocytes from healthy volunteers (age, 50-60) (A) and from pre-cancerous patients (age, 50-60) (B), after treating the different treatment groups: NC (untreated cells), excipient mixture (EM)(0.1%), MYR B (10-40µM), MYR N (10-40µM), PC (H_2O_2 50µM) for 1, 24 and 48 hours measured using the MTT assay. The percentage cell survival was evaluated for various concentrations of the test chemicals, while compared to the untreated group (NC). The values represent the mean of 3 separate experiments. (ns=not significant, $^{*}P<0.01$, $^{**}P<0.003$)

3.3.5 Viability of lymphocytes

The viability is calculated as the number of live/viable cells over the total number of cells present within the haemocytometer's grids. Non-viable cells take up the trypan blue whereas viable cells disregard trypan blue dye due to the cells intact membrane (Henderson et al., 1998). Various concentrations of all chemicals used in this study were tested for their cytotoxicity before and after drug treatment. Cells which expressed viability ≥75% were used for the study. The concentrations of flavonoid used (determined by dose response curve) were non-toxic to the lymphocytes and the vehicle substance, excipient mixture (EM) used throughout the study had no effect on the DNA damage. Neither of the chemicals had a significant effect on lymphocyte cell viability at different time periods when compared to the untreated group (NC) (figure 3.4).

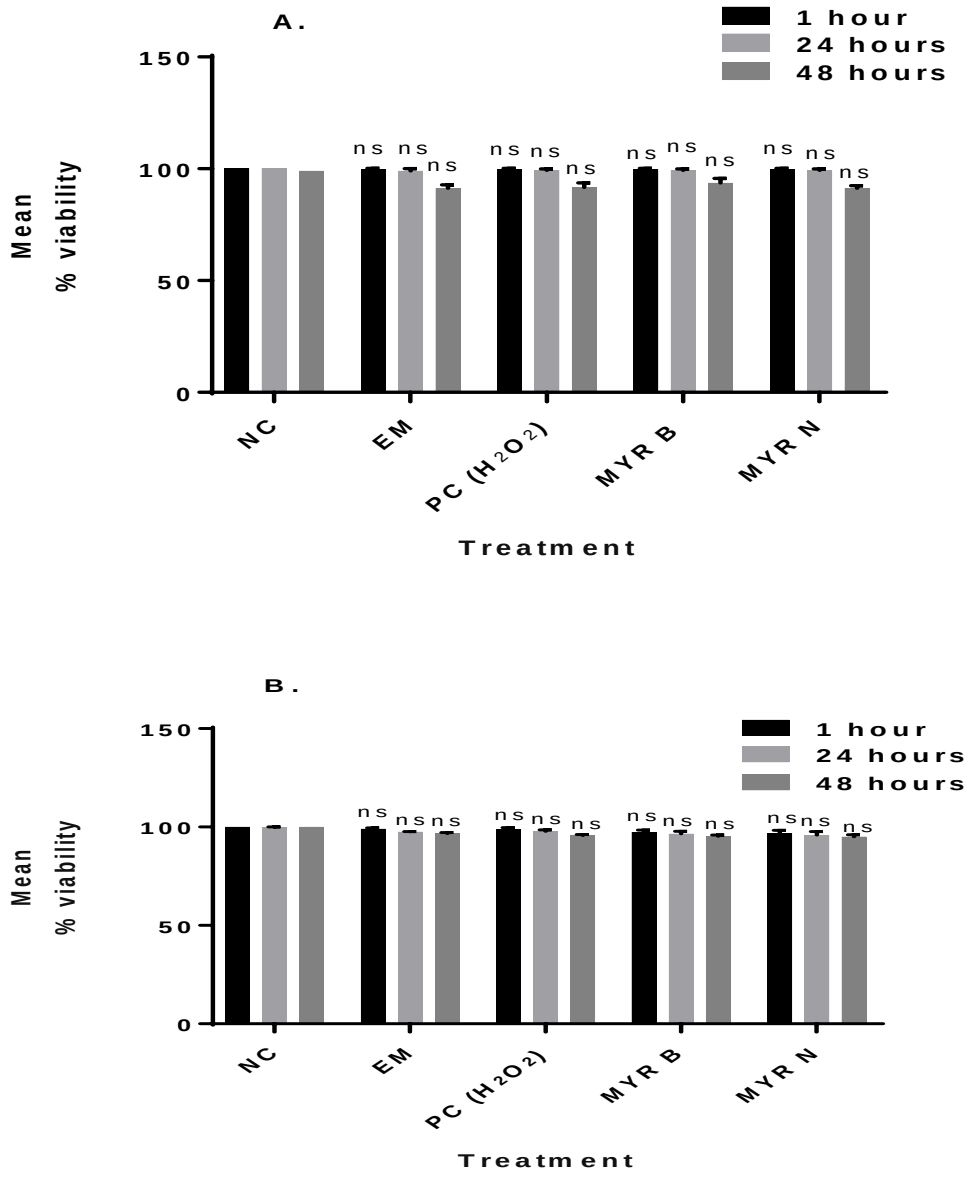


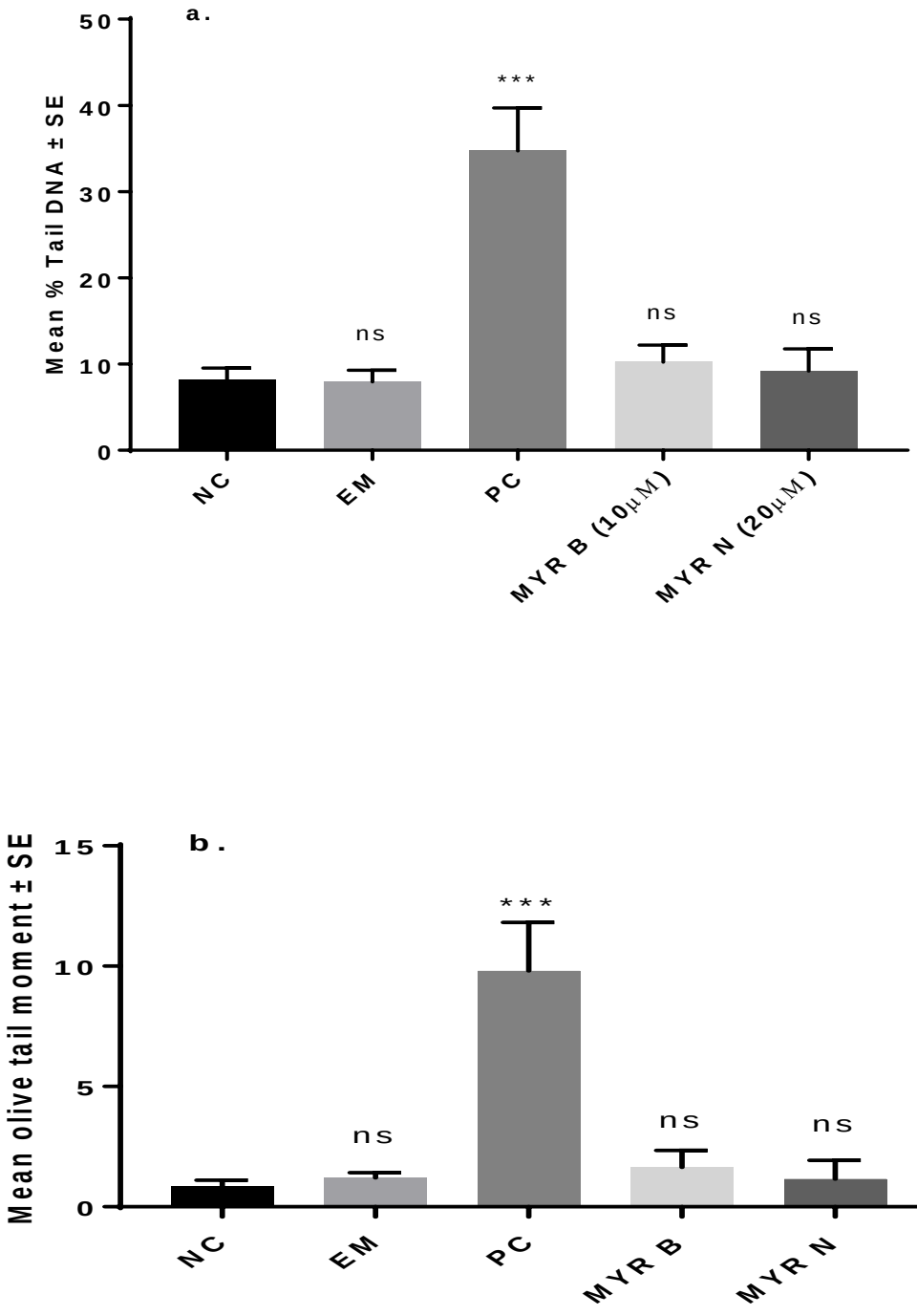
Figure 3.4 Shows mean % viability of lymphocytes from healthy individuals (age, 50-60) (A) and pre-cancerous patients (age 50-60) (B) from 3 independent experiments counting (100 cells each/treatment) using trypan blue dye. Treatment groups used were untreated (NC), excipient mixture (EM)(0.1%), positive control (PC) H_2O_2 50µM, MB (10 µM) and MN (20 µM). There was no significant difference on viability of cells on different time periods when compared to respective NC group. Ns=not significant

3.3.6 Assessment of DNA damage by using the Comet Assay

3.3.6.1 Direct comparison between the effects of myricetin nanoparticles and bulk forms on DNA damage of lymphocytes from healthy volunteers

After treating lymphocytes from healthy individuals with MYR B (10µM) and MYR N (20µM) using the Comet assay, there was no significant effect observed

in DNA damage when compared against the untreated control. However, the PC induced significantly levels of genotoxicity (Figure 3.5a, b).



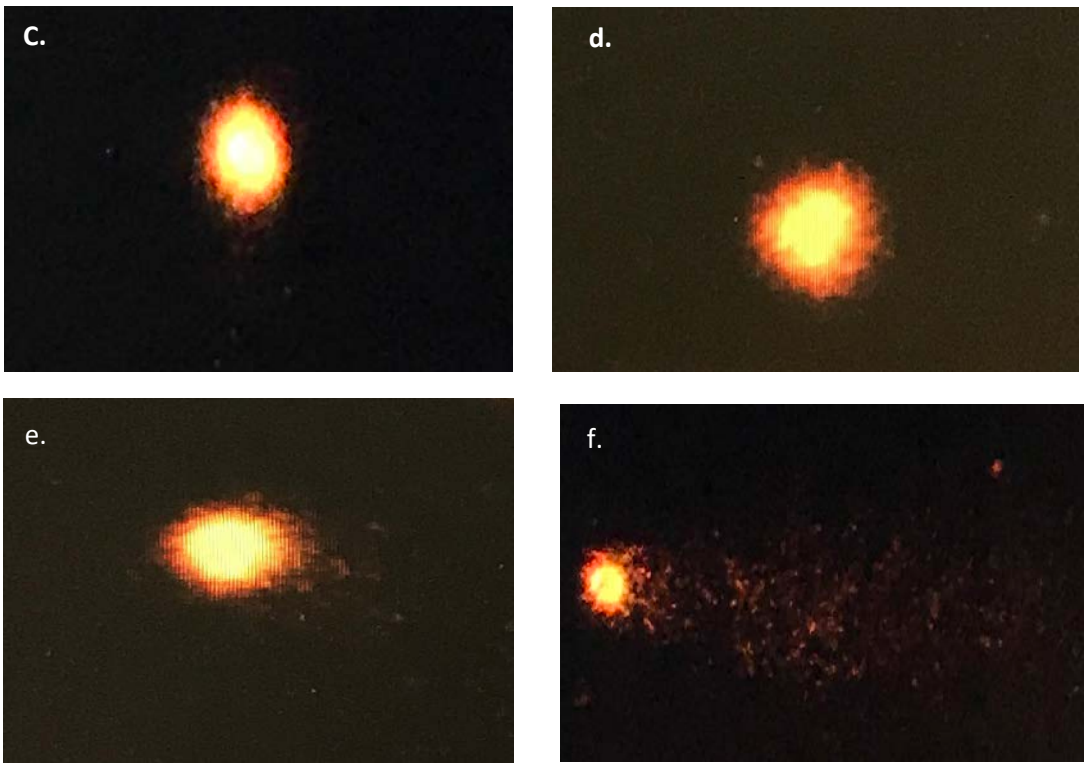
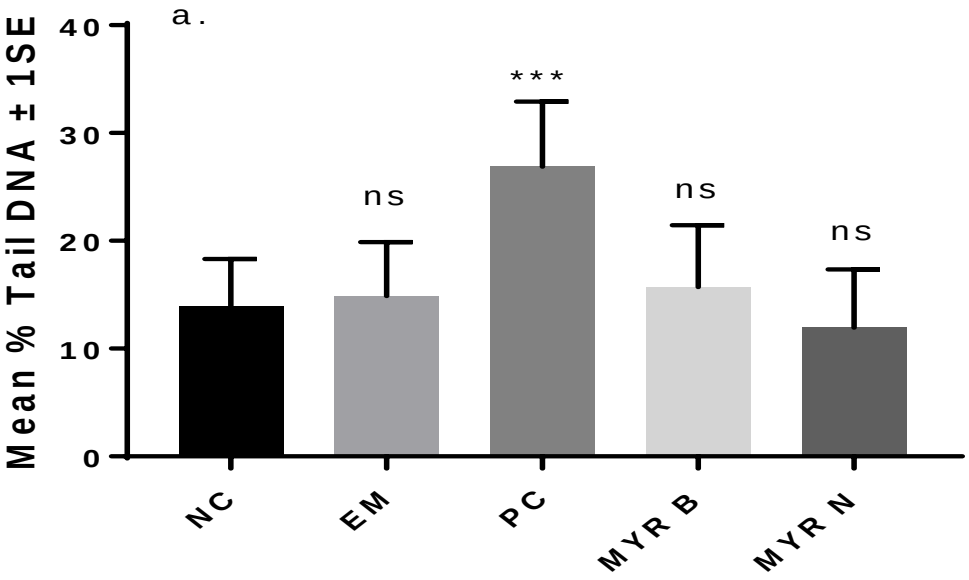
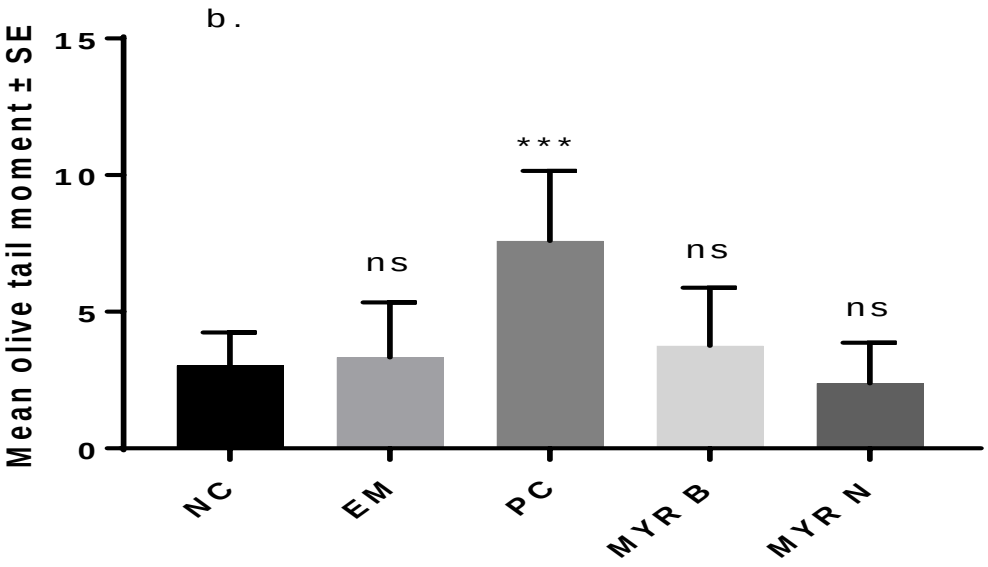


Figure 3.5 (a) The response of bulk and nano forms of myricetin on lymphocytes' DNA from healthy individuals using %Tail DNA. The figure shows the mean of experiments in 20 individuals, counting 100 cells each for four different groups of treatments; an untreated lymphocyte group (NC), excipient mixture (EX), positive control (PC) 50μM H_2O_2, myricetin bulk (MYR B 10μM) and myricetin nano (MYR N 20μM). All treatment groups were compared to the NC group. The mean control value was 8 and the PC had the maximum mean value of 34 for % Tail DNA.(****P<0.001,ns means not significant). **(b) The response of bulk and nano forms of myricetin on lymphocytes DNA from healthy individuals using Olive tail moment (OTM).** The mean OTM of four treatment groups from experiments in 20 individuals is plotted in the graph with 100 cells counted for each. Treatment groups include the negative control, excipient mixture (EX), the positive control (50μM H_2O_2), myricetin bulk (MYR B 10μM) and myricetin nano (MYR N 20μM). While comparing to the negative control both MYR N and MYR B did not show any significance difference. The mean control value of OTM was 0.8 and that for PC was 9. (***P< 0.001, ns represents not significant). **(c)** Control lymphocyte. **(d)** MYR N (20μM) treated lymphocyte. **(e)** MYR B (10μM) treated lymphocyte. **(f)** H_2O_2 (50μM) treated lymphocyte cell showing DNA damage in Comet tail at mag X20.

3.3.6.2 Comparison between myricetin nano and bulk form responses on DNA of lymphocytes from pre-cancerous patients

In vitro treatment of lymphocytes taken from pre-cancerous patients with MYR B (10μM) and MYR N (20μM) showed no significant difference from the control group. (Figure 3.6a, 3.6b)


a.
Mean % Tail DNA ± 1SE
40
30
20
10
0
***
ns
ns
ns
NC
EM
PC
MYR B
MYR N


b.
Mean olive tail moment ± SE
15
10
5
0
***
ns
ns
ns
NC
EM
PC
MYR B
MYR N

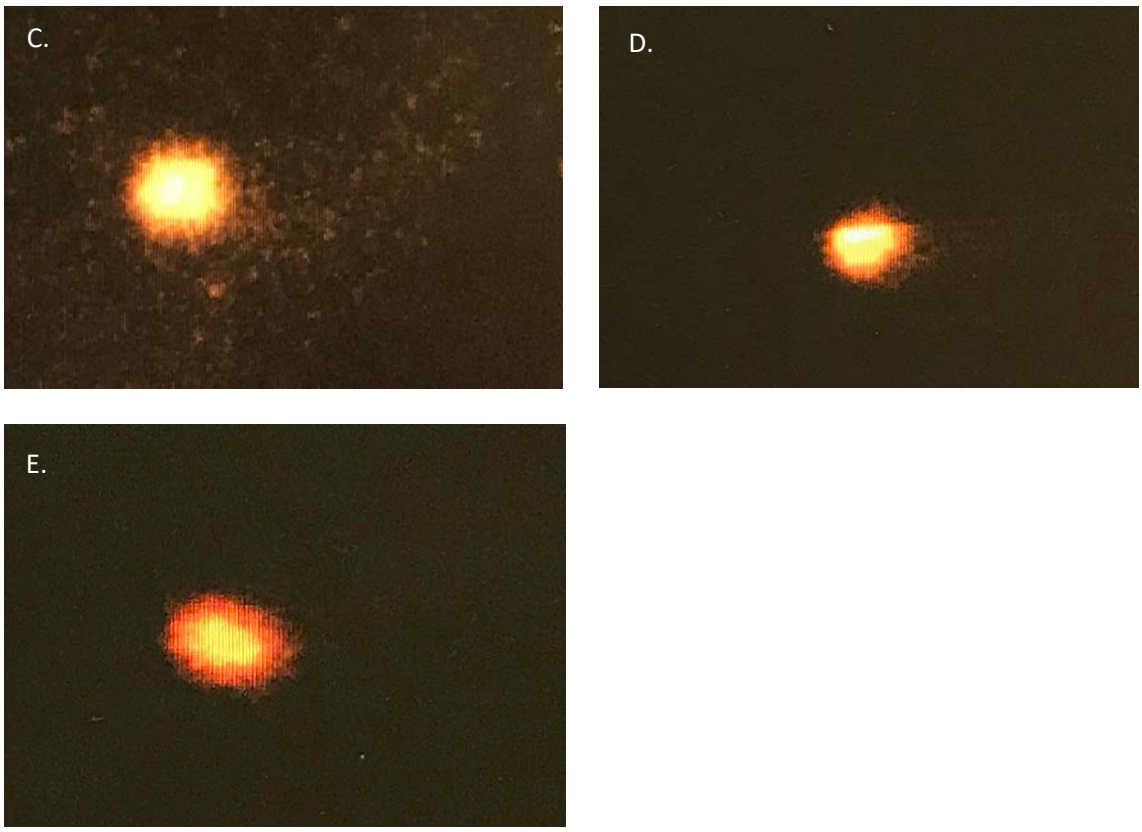


Figure 3.6 a The response of MYR B and MYR N, on lymphocytes from pre-cancerous patients using % Tail DNA. This graph shows the mean and standard errors of experiments on 20 individuals (measuring 100 cells each) in which four treatment groups were tested for DNA damage in patient lymphocytes as follows: an untreated lymphocyte group (NC), excipient mixture (EX), positive control (PC) 50µM H_2O_2, myricetin bulk (MYR B 10µM) and myricetin nano (MYR N 20µM). All other groups were compared to the negative control. The mean negative control and positive control values for % Tail DNA were 14 and 26, respectively. (***P< 0.001, ns represents not significant).**b Response of MYR B and MYR N, on pre-cancerous patient lymphocyte using Olive tail moment.** The data is expressed as mean and standard errors of experiments on 20 individuals, where dose dependent effects of the excipient mixture (EX), the positive control (50µM H_2O_2), MYR B (10µM) and MYR N (20µM) were compared against the negative control group on lymphocytes from the patient group. (***P< 0.001, ns means not significant). **(c)** Untreated lymphocyte from pre-cancerous patient. **(d)** MYR N (20µM) treated lymphocyte from pre-cancerous patient. **(e)** MYR B (10µM) treated lymphocyte from pre-cancerous patient at mag X20

3.3.7 Determination of micronuclei (MNi) and other DNA damage

3.3.7.1 Introduction

Treatment of lymphocytes from healthy volunteers with MYR B and MYR N has induced MNi in binucleated cells (BiNC) when compared to the controls but the induction was not significant. In patient lymphocytes, MNi frequency in BiNC treated with MYR B was the same as the negative control. After treatment of

healthy lymphocyte with MYR B, the MNi number in mononucleated cells (MoNC) increased but there was no difference seen with MYR N, when compared to the negative control group. MNi frequency in MoNC seemed to reduce in patient lymphocytes with both forms.

3.3.7.2 MNi frequency in BiNC

The number of MNi in BiNC from pre-cancerous patients (aged between 55 and 65) was higher than those from healthy individuals (aged between 45 and 60). MYR B and MYR N have induced MNi formation in healthy lymphocytes at a non-significant level. However, in patient lymphocytes, both forms of myricetin have shown a trend towards lowering the induction of MNi in BiNC when compared to respective untreated group. Again the levels were not significantly different (Figure 3.7a).

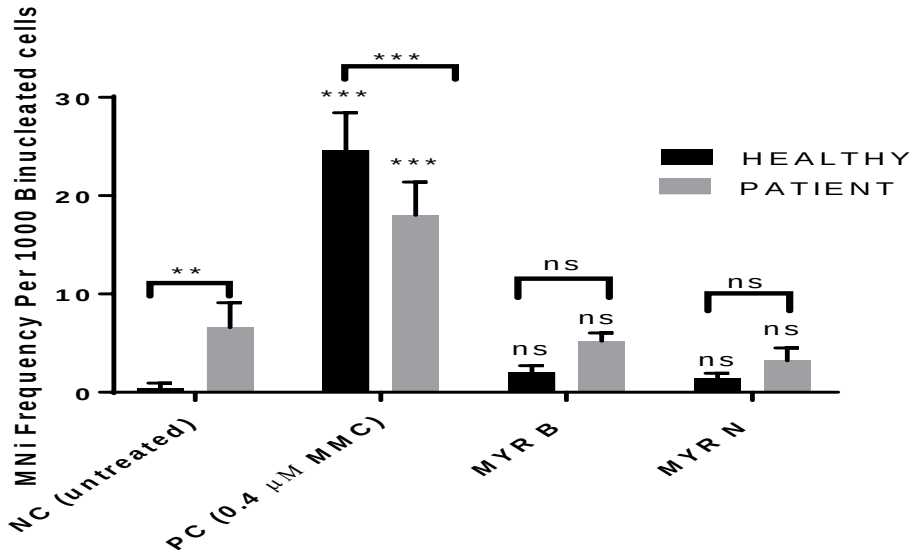


Figure 3.7a Shows the average of BiNC MNi scored per 1000 cells per culture from 5 independent experiments, n=1000. Data are expressed as means ± standard deviations (SD). * Shows significant difference between the groups, ns = not significant. Four treatment groups included the negative control, a positive control group (Mitomycin C {0.4μM MMC}), MYR B (10μM) and MYR N (20μM) group.(*** represents P< 0.0001, ** represents P< 0.01). Horizontal lines with the statistics above them show the differences between the groups. The statistics below horizontal lines show the comparison of treatment groups with respective untreated group.

3.3.7.3 MNi frequency in MoNC

Again the frequency of MNi in MoNC from patient's lymphocytes was higher than healthy group at basal levels. Mitomycin significantly induced MNi formation in both groups. MYR B has not shown significant effect on MNi induction on either of the groups. However, MYR N has reduced MNi formation MoNC from pre-cancerous patients. There are significant differences between the two groups (healthy and patient) for both MYR B and MYR N treatments which have been shown using the horizontal bars in the graph below (figure 3.7b).

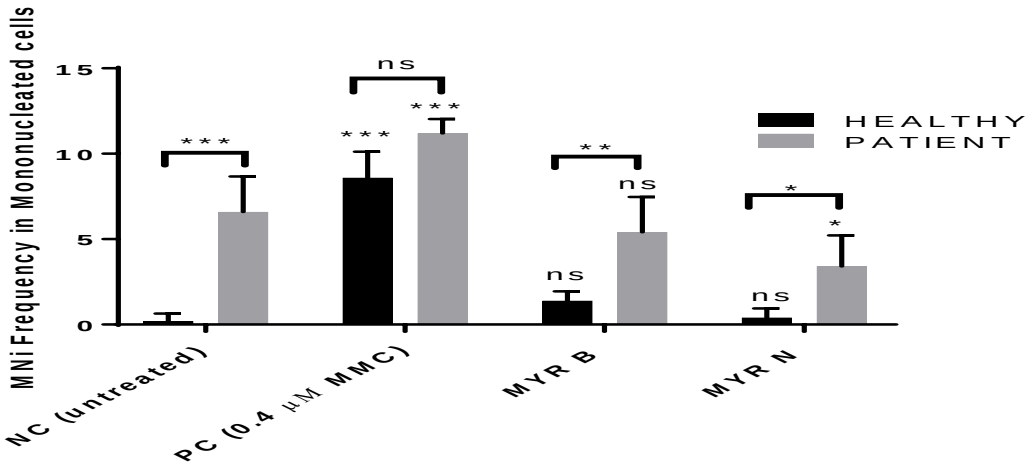


Figure 3.7b Shows, MNi frequency observed in MonoNC cells. Average of 5 independent experiments in 5 individuals for each healthy and patient group. Treatment of lymphocytes with the positive control (Mitomycin C {0.4µM MMC}), MYR B and MYR N, compared to the respective untreated groups. Stars represent significant differences. (***P< 0.001,* P< 0.02 and **P< 0.006). Horizontal lines with the statistics above them show the differences between the groups. The statistics below horizontal lines show the comparison of treatment groups with respective untreated group.

3.3.7.4 Other determinants of MN

The following table (3.6) shows the other important biomarkers of damage assessed in the micronucleus assay. There is no significant difference between the NDI and BiNC percentage among all the treatment groups. Untreated cultures of patient group shows higher number of MNi both in MoNC and BiNC

as compared to the groups treated with MYR B (10µM) and MYR N (20µM). MYR B and MYR N have shown trend to reduce MNi regardless of group difference and cell type but the values are not significantly different except for MYR N (20µM) which has significantly reduced MNi formation in MoNC from pre-cancerous patients (Statistics presented in Fig 3.7a and b). EM did not cause any damage in healthy lymphocytes. However, MNi formation seen in lymphocytes from pre-cancerous patients is lower than the respective untreated group which indicates that EM does not induce MNi or any other chromosomal damage. MYR B and MYR N at selected non-genotoxic concentrations did not induce any nuclear bridges or buds in both investigative groups.

Subject	Treatment Group	NDI	% BiNC	Per 1000 BiNC CELLS			% MNi in MoNC
				BiMNi	BiNPB	BiBuds	
Healthy individuals	Untreated lymphocytes	1.85	62	0	0	0	0
	0.4 µM MMC	1.73 (P<0.01)	58 (ns)	24 (P<0.0001)	5 (P<0.001)	3 (P<0.01)	8 (P<0.001)
	EM	1.84 (ns)	60 (ns)	0 (ns)	0 (ns)	0 (ns)	0 (ns)
	MYR B	1.82 (ns)	63 (ns)	2 (ns)	0 (ns)	0 (ns)	1 (ns)
	MYR N	1.85 (ns)	64 (ns)	1 (ns)	0 (ns)	0 (ns)	0 (ns)
Pre-cancerous patients	Untreated lymphocytes	1.81	61	6	1	0	6
	0.4 µM MMC	1.85 (ns)	61 (ns)	18 (P<0.001)	4 (P<0.04)	2 (P<0.05)	11 (P<0.001)
	EM	1.80 (ns)	62 (ns)	4 (ns)	0 (ns)	0 (ns)	6 (ns)
	MYR B	1.84 (ns)	64 (ns)	5 (ns)	0 (ns)	0 (ns)	5 (ns)
	MYR N	1.87 (ns)	62 (ns)	3 (ns)	0 (ns)	0 (ns)	3 (P<0.02)

Table 3.6 The average of various markers of chromosomal damage in micronucleus assay. Showing NDI per treatments on healthy and patient cells, mean % of BiNC, mean number of MNI,NPBs and NBUDS per 1000 BiNC and MNi frequency in MoNC. Five treatment groups included the negative control, a positive control group (Mitomycin C {0.4µM MMC}), excipient mixture (0.1 %) (EM), MYR B (10µM) and MYR N (20µM). ns=not significant

3.3.8 Myricetin activates the P53 signalling pathway independent of DNA damage in lymphocytes

Based on our previous results from the Comet and micronucleus assays we established that MYR B (10μM) and MYR N (20μM) have shown anti-genotoxic effects in lymphocytes from healthy individuals and pre-cancerous patients. MYR N (20μM) exhibited genoprotective effects in lymphocytes from pre-cancerous patients by significantly reducing the MNi induction in MoNC. To identify the molecular mechanisms involved in this effect, we studied the influences of MYR B and MYR N on the gene expression levels of P53, a tumour-suppressor multi-functional gene in patients versus healthy lymphocytes and ATM kinase mRNA in patient lymphocytes. Tumour-suppressor P53 gene encodes for proteins that prevent genome mutations by interacting with DNA and regulating gene expression. Thus, plays significant role in prevention of carcinogenesis. It is most frequently mutated gene in various cancers (Surget et al., 2013). ATM kinase also plays a crucial role in maintenance of genome stability and facilitates DDR, initiating many physiological responses particularly in reaction to the formation of DNA DSBs; hence, it is used as biomarker of DSBs (Boohaker and Xu, 2014). This is why we studied P53 and ATM at the gene level. Total RNA was isolated from lymphocytes pre-treated with both forms of myricetin and subjected to quantitative real-time PCR analysis. The results (fig 3.8a) have shown that myricetin bulk has induced non-significant upregulation of P53 gene in lymphocytes from both groups, healthy and patients. However, myricetin nanoparticles significantly up-regulated the gene expression of P53 in patient lymphocytes compared to those from healthy individuals. This indicates that the protective effects caused by myricetin might be dependent on the tumour-suppression activity of the P53 gene. The ATM

gene expression has not shown significant effect in lymphocytes from both groups when treated with MYR B and MYR N (fig 3.8b).

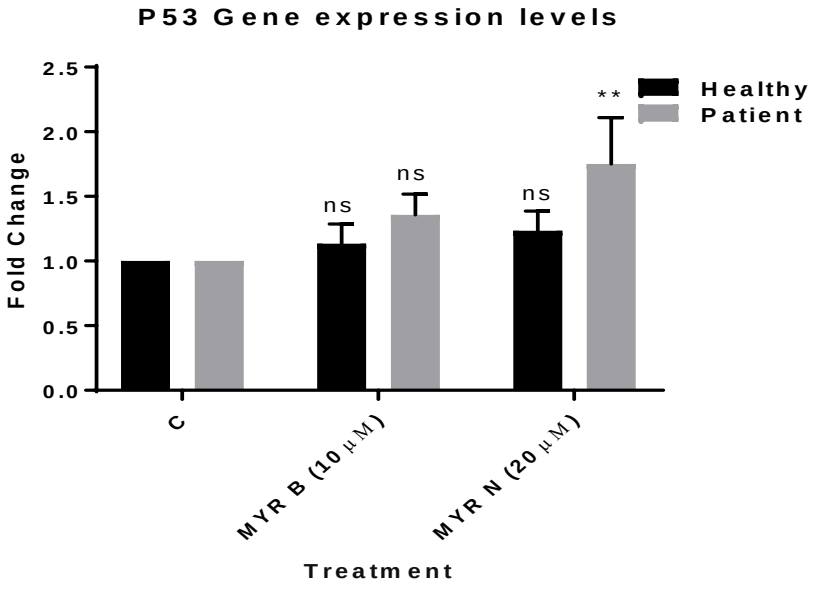


Figure 3.8a The influence of MYR B and MYR N, on the expression of P53 mRNA in lymphocyte from healthy individuals and pre-cancerous patients. GAPDH was used as an internal control gene. Gene expression analysis was performed on lymphocytes after 24-hour treatment. P53 mRNA expression was significantly increased in lymphocyte from pre-cancerous patient after exposure to MYR N. Values are the means ± standard errors (SE) of three independent experiments, and the error bars represent SDs. The p values are **p=0.008, ns=not significant. All values were compared against respective control (C) and normalised against GAPDH reference gene.

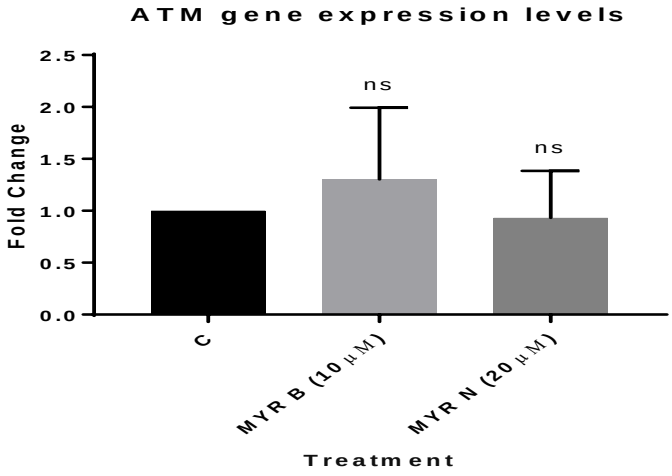
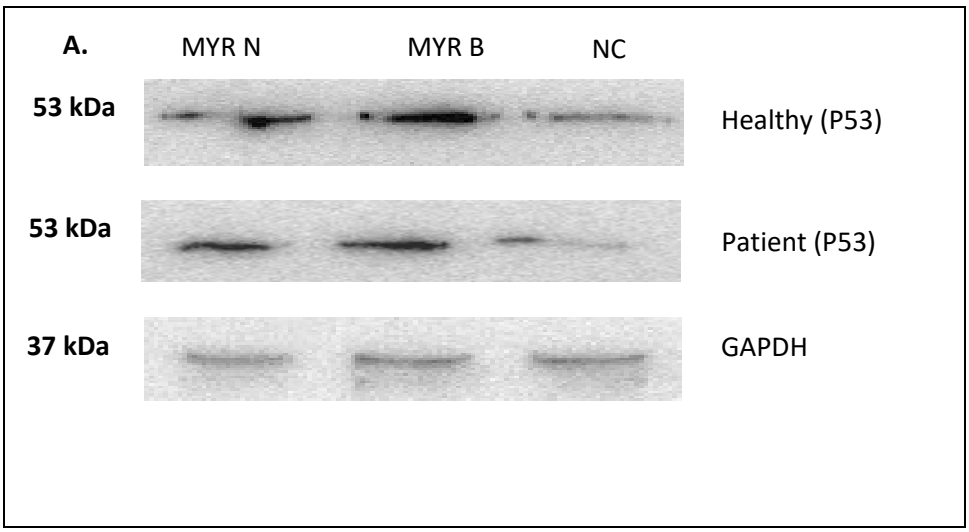


Figure 3.8b The influence of MYR B and MYR N, on the expression of ATM mRNA, in lymphocyte from pre-cancerous patients. GAPDH was used as an internal control gene. Gene expression analysis was performed on lymphocytes from pre-cancerous patients after 24-hour treatment. ATM mRNA expression was increased in lymphocyte treated with MYR B but values are not significant. Values are the means of three independent experiments/individuals, and the error bars represent SDs. ns=not significant. All values were compared against control (C) and normalised against GAPDH reference gene.

3.3.9 Analysis of p53 protein expression in lymphocytes

P53 plays a vital role in regulation of various cellular outcomes and responses such as angiogenesis, cell cycle arrest in the presence of DNA damage, DNA repair, apoptosis and transcription (Shaw, 1996; Haupt and Haupt, 2017). As cell cycle arrest and DNA repair are essential processes to protect cells against further DNA damage and pre-existing damage, we investigated the effects of myricetin nanoparticles and bulk forms on P53 protein expression in the lymphocytes from pre-cancerous patients compared to those from healthy subjects. We carried out Western blot procedure and analysed protein expression in lymphocytes from three healthy and three pre-cancerous patient samples. We restricted our study to only three experiments for each experimental group due to limited patient blood samples. The results obtained were consistent with our PCR data. P53 protein expression is significantly increased by 2.3-fold ($P<0.0001$) with MYR B ($P<0.0001$)) and 2.0-fold with MYR N treatment in healthy lymphocytes (fig 3.9 A, B). Also, p53 showed a 1.3-fold increase in the lymphocytes of pre-cancerous patients treated with MYR B, a 1.4-fold-increase ($P=0.0140$) after treatment with MYR N ($P=0.0036$).



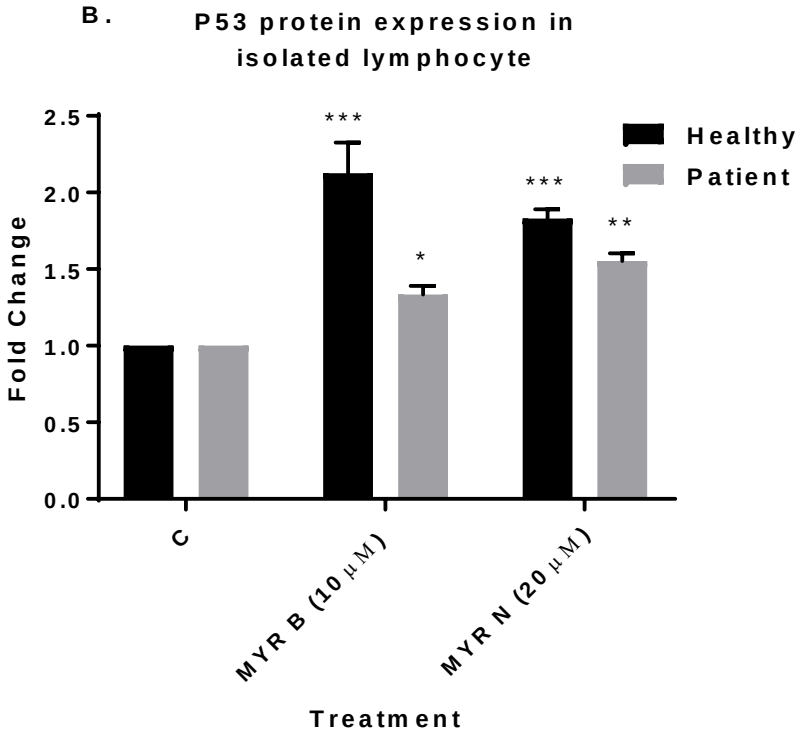


Figure 3.9 Myricetin Bulk and nanoparticles significantly up-regulate the P53 protein levels in lymphocytes from pre-cancerous patients and healthy individuals (A) Immunoblot analysis of the p53 protein in lymphocyte treated with MYR B and MYR N. P53 expression was increased to 2.3-fold by MYR B and 2-fold by MYR N in the healthy group. In pre-cancerous patient MYR B induced 1.3-fold increase and MYR N treatment caused 1.4-fold increase in P53 expression. GAPDH was used as an internal control protein to normalise the data. **(B)** Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SEM of three experiments. $^{***}P<0.0001$, $^{**}P=0.0036$, $^{*}P=0.0140$

3.4 Discussion

The current *in vitro* study investigated the effect of myricetin bulk and nanoparticle forms on human lymphocytes from pre-cancerous patients and those from healthy individuals.

Various *in vivo and in vitro* studies have demonstrated the promising health benefits of myricetin including anti-inflammatory, anti-oxidative, anti-proliferative, cytoprotective effects, anti-carcinogenic, anti-viral and anti-microbial properties. Research has shown that myricetin inhibits the growth of human promyelocytic leukaemia (HL-60) cells by induction of apoptosis

(Hibasami et al., 2005), decreases the viability of HL-60 cells via apoptosis through a ROS independent and mitochondria dependent pathway (Ko et al., 2005) and induces pancreatic cell death *in vitro* (Phillips et al., 2011). Research has also indicated that myricetin is a strong inhibitor of the prostate cancer cell line PC-3 (Xu, 2013). However, none of the previous studies have tested *in vitro*, the effect of myricetin bulk and nano forms on the DNA of human lymphocytes from pre-cancerous patients. Genetically compromised DNA repair mechanisms might contribute to raised levels of DNA damage in lymphocytes from patients (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011) hence; lymphocytes are the focus of the current study. Peripheral lymphocytes represent an excellent model for examining the genome sensitivity (a factor of susceptibility to cancer) (Collins, 2004). If other somatic cell types are not accessible then lymphocytes can be used as surrogate cells to investigate the DNA damage and lymphocytes circulate in the blood stream across the body hence; are vulnerable to both endogenous and exogenous DNA damage by physio-chemical genotoxic insults (Najafzadeh et al., 2009). The alkaline Comet assay has been used to assess the DNA damage in lymphocytes from leukaemia patients (Collins, 2009). It has been proven a reliable method to assess the genotoxicity of NPs (Azqueta and Dusinska 2015).

The Comet assay results from our study show that exposing lymphocytes from healthy individuals and pre-cancerous patients to myricetin nano and bulk at lower concentrations (20µM and 10µM, respectively) did not induce any significant DNA damage when compared to their respective negative control groups (Fig 3.5a, b and 3.6a, b). The anti-genotoxic behaviour of MYR B and MYR N at selective lower concentrations of 10µM and 20µM, respectively shown in this research is consistent with an earlier study which has shown that

myricetin is a non-toxic and anti -mutagenic flavonoid (Wang, 2010). However, myricetin has induced dose dependent genotoxicity at higher concentrations which might be possible due to the fact that flavonoids can potentially exhibit various activities at slightly different concentrations depending on the stimulus and environment they interact with. Therefore we only used non-genotoxic concentrations of both forms of myricetin to avoid any artefact due to toxicity. Due to the wide range of physio-chemical properties of NPs, various characteristics (shape, size, solubility, composition, surface properties, stability, uptake and aggregation) of these compounds need to be considered to be able to assess their genotoxic effects, but the specific properties responsible for genotoxicity, are still uncertain. These properties have direct impact on the biological activities of NPs (Chan, 2006). The unique chemical composition of myricetin containing hydroxyl group at positions 3, 5 and three consistent hydroxyl groups at 3', 4' and 5' positions can enhance the effectiveness of its antioxidant behaviour (Li and Ding, 2012).

DNA damage can occur as a result of primary and secondary genotoxic or mutagenic events. This can be characterized as DNA adducts, DNA base modifications, single and double strand breaks, structural DNA changes or cross links (Karlsson et al., 2008). This damage can be repaired by various repair systems: base excision repair (BER), nucleotide excision repair (NER), homologous recombination or non-homologous end joining (NHEJ). If the DNA damage is not repaired by any of these possible mechanisms, it can cause mutations which can eventually lead to cancer development. Hence, repairing the DNA and chromosomal damage is crucial for sustenance of particular organ or system (Sharma et al., 2012).

The cytokinesis-block micronucleus assay, a test, to determine the capability of genotoxic substance to induce aneugenic and clastogenic effects on cell cycle and cellular division (Fenech, 2002) was used in the present study, for the first time to investigate the effects of myricetin bulk and nano forms on lymphocytes, particularly from pre-cancerous patients. A micronucleus (MN) is formed during anaphase and could represent chromosomal fragments or lost chromosomes when the nucleus divides (Magdelenova et al., 2012). The sensitivity of the assay is enhanced by cytochalasin B to block the cytokinesis, but not the mitosis, to facilitate binucleated cells accumulation. The MN in binucleated cells only shows the damage caused after the treatment and reduces the probability of scoring the pre-existing damage (Magdelenova et al., 2012). Mitomycin C (MMC), known as a clastogen, genotoxic and anti-cancerous agent, was used as a positive control as its already experimented in our laboratory and it worked as expected by inducing MNi typically in binucleated cells rather than mononucleated (Elhajouji et al., 1998). Lower mean NDI and lower mononucleated cells were observed by treatment with MMC, which is consistent with previous studies performed at this laboratory (Najefzadeh, 2012).

The normal range of BiNC and NDI in the healthy control is 30-60% and 1.3-2.2 respectively. Variations in these readings could be due to confounding factors: age (more MNi in older people), gender (MNi are common in females) or simple lifestyle factors (smoking, illness, drinking and medications etc.) along with concentrations of the drug under test (Fenech, 2007; Houhannisyan et al., 2009).

Most of the NDI for the treatment groups were within the normal range but a few were elevated possibly because of other factors but were not significantly raised

indicating the division (Table 3.6). Evaluation of the assay shows that the frequency of MNi in MoNC from pre-cancerous patients significantly decreased when treated with MYR N (20µM) but not with MYR B (10µM), compared to the untreated group. This may be possibly due to either van der Waals forces or electrostatic interaction of NPs with nuclear proteins for example repair proteins and facilitated repair (Magdelonova, 2012). The double concentration of MYR N compared to MYR B could also contribute towards its effective behaviour, and in moving this work forward it would be important to directly compare MYR N and MYR B at both 10µM and 20µM. Also, the potential explanation for this is that NPs due to their very small size can easily reach the nucleus through diffusion across the nuclear membrane or transportation via the nuclear pores and gain direct interaction with the DNA (Magdolenova et al., 2014). The shape of NPs also plays a vital role in their internalisation and penetration into the cells (Guo et al., 2011). NPs can also enter the cell by endocytosis and eventually gain access to nuclear DNA (Chen et al., 2013). As the size of NPs decreases, the surface area and the numbers of atoms on the surface enormously increase and this makes NPs more reactive in biological systems. This property enhances their dispersion across the body and some can enter individual cells (Chan, 2006; McNeil, 2005). Different surface changes including charge and chemistry, enable the attachment of NPs to molecular, chemical or other biological bodies. This results in their different behaviours in solution (McNeil, 2005). The solubility and stability of NPs also affect their activity by affecting their bioavailability in living systems (Franklin et al., 2007).

Myricetin in both forms did not induce any nuclear bridges or buds. There were few MNi in MoNC seen in healthy lymphocytes treatment which indicates little pre-existing DNA damage due to lifestyle factors or any other medical

procedure carried out. However, this number was raised in pre-cancerous patients due to increased levels of basal damage.

Several studies have focused on understanding the cellular mechanisms involved in controlling the cell cycle changes, in reaction to DNA damage (Kastan et al., 1992; Kuerbitx et al., 1992). The critical molecular role played by P53 in DNA damage induced-cell cycle arrest is well distinct (Kastan et al., 1991). Upon sensing the DNA damage P53 gene up-regulate the expression of $p21^{WAF1}$ gene which then inhibits the activity cyclin CDK complexes leading to G_1 arrest blocking the progression of cell cycle and transcription. The down-regulation of cyclin A expression initiated by P53 could stop the entry into and through S phase. Without P53 normal function, DNA integrity could be compromised causing high mutational rates (Shaw, 1996). It has been well documented that P53 stimulated $p21^{WAF1}$ interacts with a complex called proliferating cell nuclear antigen (PCNA), involved in inhibition of replicative DNA synthesis (Waga et al., 1994) yet promoting the excision repair mechanism (Shivji et al., 1994). Mutations in P53 genes are the most common genetic lesions found in all types of human tumours leading to the uncontrolled proliferation of tumour cells and cell cycle progression. However, a complete loss of normal P53 function is required for a tumour to develop (Ventura et al., 2007).

Evident from previous work on P53 and its various functions in maintaining the cellular integrity we understand that by increasing the amount of P53, cellular proliferation of tumour cells may be suppressed through DNA repair, cell cycle arrest and apoptosis. This could be a new strategy for treating and preventing cancer development. Our results demonstrate that 24-hour treatment with both

the nanoparticles and bulk forms of myricetin activates and stabilises the p53 protein at the post-translational level (Figure 3.9), suggesting that myricetin may ensure p53-dependent action—for instance, cell cycle arrest and DNA repair in pre-cancerous patients.

ATM deletions or mutations are linked with the development of periodic human cancers, for instance leukaemia and mantle cell lymphoma (Stilgenbauer et al., 1997; Schaffner et al., 2000). Previous studies proposed that the ATM gene express a protein that detects DNA damage and directs signals to the cells for stimulating apoptosis (Shiloh, 1997; Marinoglou, 2012). Research also indicates the ability of ATM kinase to trigger defence systems against oxidative injury and reduce DNA damage by inhibiting ROS (Chen et al., 2003; Semlitsch et al., 2011; Shiloh, 2014).

However, our results have not shown any significant change in ATM gene expression levels in lymphocytes from pre-cancerous patients after exposure to myricetin bulk and nano forms (figure 3.8b).

3.5 Conclusion

Our results from the Comet and micronucleus assays showed that MYR B (10μM) and MYR N (20μM) did not induce significant DNA damage in the lymphocytes from healthy individuals and pre-cancerous patients. However, both forms of myricetin do induce concentration dependent genotoxicity at higher concentrations. MYR N (20μM) has shown genoprotective effects in lymphocytes from pre-cancerous patients by significantly inhibiting MNi induction in MoNC. This could be possible due the double concentration used for MYR N than MYR but also due to the enhanced properties of NPs including the effectiveness of diffusing through the biological membranes and interacting

directly with nucleus. These findings suggest that MYR B and MYR N can potentially exhibit both genotoxic and anti-genotoxic properties depending on the concentration, the stimulus applied and the substrate. However, at non-genotoxic concentration myricetin could protect the lymphocytes of healthy individuals and pre-cancerous patients against DNA damage and possibly facilitate repair through a P53-mediated pathway.

Chapter 4: Reactive oxygen species (ROS) induced oxidative damage in lymphocytes from healthy individuals and those from pre-cancerous patients: Protection by myricetin nanoparticles and bulk forms by stimulating endogenous anti-oxidative defence mechanism

4.1 Introduction

Oxidative stress is a major factor contributing towards the development of various illnesses and it is a key inducer of cellular lesions. It is triggered by a disparity between the production of ROS and the capacity of biological system to cleanse the reactive intermediates. Mitochondria are the main supply of intracellular ROS and ROS have been considered as a second carrier and partial participant in different physiological processes including proliferation and apoptosis. ROS have been shown to induce proliferation in tumour cells and mediate the proliferation initiated by epidermal growth factor (EGF) or platelet derived growth factor (PDGF) (Burdick et al., 2003; Chao-Wei Chen et al., 2004). Thus, anti-oxidants show an effective preventative effect against cancer development by reducing the ROS levels and inhibiting their production. In addition, intracellular ROS production has been associated with the mediation of the effects caused by anti-cancer drugs like taxol (Perkins et al., 2000; Varbiro et al., 2001). Hence data suggests a two-sided role of ROS. It has been suggested that cellular redox potential may be regulated through increasing glutathione (GSH) pools to the mitochondria, hence, preventing ROS formation and cellular injury caused by oxidative stress (Slikker et al., 2001; Imam et al., 2001).

Oxidative stress produced by tertiary-butyl hydro peroxide (TBHP) and hydrogen peroxide (H_2O_2) is well documented. TBHP is a toxic compound and causes extreme discomfort to various organs and systems (Zavodnik et al., 1988; Sarkar and Sil, 2010; Bhattacharya et al., 2011). Exposure of cellular components to TBHP results in an increase of membrane permeability along with hyperpolarization. Reaction of TBHP with haemoglobin forms t-butoxyl radicals, which then initiate peroxidation by interacting with membrane lipids

(Deuticke et al., 1987; Zavodnik et al., 1988)). Extensive lipid peroxidation leads to membrane disturbance (Benatti et al., 1982). Cellular antioxidant enzymes like glutathione, to some level inhibit membrane disruption by scavenging the t-butoxyl radicals.

Myricetin (3,5,7,3',4',5'-hexahydroxyflavone), an important flavonol, is widely found in berries, fruit and vegetables (Wang et al., 2010). A large body of data has been published regarding the anti-oxidant potential of myricetin, leaving no doubt that the compound is a strong anti-oxidant. It depicts anti-oxidant activity by scavenging reactive oxygen species (ROS) through oxidation of the three hydroxyl groups (3',4',5'-position) attached in its B ring. It may also act as an anti-oxidant by chelating redox-active metal ions such as Fe^{2+} thus stops the progression of Fenton reactions and their harmful products (Chobot and Hadacek, 2011). Studies have demonstrated that the physiological concentrations range of myricetin (5-10μM) (Peng and Kuo, 2003) could significantly inhibit the production of peroxynitrite- induced double strand breaks (DSBs) (Chen et al., 2011) and protect against the oxidative damage caused in neurodegenerative disorders (Laabich et al., 2007; Shimmyo et al., 2008).

Myricetin has been demonstrated to prevent the tertiary-butyl hydro peroxide (TBHP)-induced chemiluminescence of mouse liver homogenates (Fraga et al. 1987). Myricetin also mitigated TBHP induced oxidative stress in erythrocytes from Type-2 diabetic patients *in vitro* (Pandey et al., 2009).

H_2O_2 –induced oxidative stress and DNA strand breaks caused in human lymphocytes and human colonocyte cells have been demonstrated and shown to be reduced upon myricetin treatment (Duthie et al., 1997).

Whenever DNA damage occurs whether endogenous or exogenous, it forms DSBs. Upon formation of DSBs in DNA, hundreds or thousands of H2AX

proteins present in chromatin are rapidly phosphorylated in the position Ser139 by kinases such as ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) in the PI3K pathway, adjoining the break site, providing signalling within the DNA DSB repair (Kuo and Yang, 2008). The newly phosphorylated derivative of H2AX is called gamma-H2AX. Measuring DSBs in human lymphocytes by immunolabelling the gamma-H2AX foci is an established method (Löbrich et al., 2005; Rogakou et al., 1998; Rogakou et al., 1999). The number of foci therefore could be used as a relative parameter to estimate DNA damage and repair.

Although myricetin is a well-established antioxidant, little is known about its beneficial role in countering the TBHP induced cytotoxicity in lymphocytes. The aim of the present study was, therefore, to investigate the *in vitro* protective role of myricetin nanoparticles (MYR N) and myricetin bulk (MYR B) against TBHP induced oxidative damage in lymphocytes from healthy individuals and pre-cancerous patients. The effect of myricetin nano and bulk forms against H_2O_2 induced damage, change in intracellular ROS due to oxidative stress caused by TBHP and change in GSH levels was also investigated. The study also assessed the effect of myricetin nano and bulk forms on DSBs by analysing the gamma H2AX protein expression immunocytochemically.

4.2 Methodology

4.2.1 Chemicals

Please refer to 2.1 (chapter 2) for details about the chemicals and materials used.

4.2.2 Blood samples

Healthy blood was collected from healthy individuals in sterile anticoagulant containing blood collection tubes. Patient blood was kindly provided by the Department of Haematology, Bradford Royal Infirmary (BRI), UK. Please refer to Table 3.1 and 3.2 for information about healthy and patients' blood samples used in current study.

4.2.3 *In vitro* experimental design

Isolated lymphocytes were maintained in complete media containing RPMI 1640 (without phenol red) with 1% penicillin-streptomycin and 15% foetal bovine serum. Different sets of lymphocyte suspensions were used for various experiments. The optimal dose for TBHP was determined by treating lymphocytes with different concentrations of TBHP followed by incubation at 37^0C in the presence of 5% CO_2. The intracellular ROS levels were assessed spectrofluorometrically after incubation with 10µl of DCFDA dye in the dark. The best incubation time for TBHP exposure was chosen as 60mins and used as standard for ROS related experiments.

The optimal concentrations of MYR B, MYR N and H_2O_2 were determined by the dose response curve for all experiments. Doses for MYR B and MYR N ranging from 10µM to 80µM were used to assess the best one. Untreated lymphocytes were set as the negative control (NC). The optimal concentrations for TBHP and H_2O_2 were used as positive controls (PC). After determining the doses for the test chemicals, PCs were simultaneously added to lymphocytes in suspension with either MYR B or MYR N in co-supplementation studies. TBHP was also considered as one of the positive control for co-supplementation

because there is not much known about the effects of Myricetin against TBHP-induced oxidative stress.

4.2.4 Cell viability and cytotoxicity

Please refer to 2.2.11 and 2.2.12 (chapter 2) for the assay procedure of trypan blue DYE exclusion and MTT.

4.2.5 The Comet Assay for determination of DNA damage

Lymphocytes were treated with MYR B and MYR N in combination with H_2O_2 (50 µM) for 1 h. The cell suspension was centrifuged at 3000 rpm (1000g). The supernatant was removed and the pelleted cells were subjected for the Comet assay as previously defined with some changes (Singh et al., 1988). Please refer to 2.2.13 (chapter 2).

4.2.6 Cell culture and measurement of ROS accumulation in lymphocytes

The fluorescent probe DCFDA was used to detect the intracellular ROS accumulation. (Refer to 2.2.16 chapter 2 for the procedure)

4.2.7 Assay of cellular enzyme and total thiol content

Reduced glutathione (GSH) is considered as the major tissue anti-oxidant. GSH levels and oxidised glutathione (GSSG) contents in experimental and normal lymphocytes were analysed using GSH/GSSG Ratio Detection Assay Kit (Fluorometric - Green) using the method described in 2.2.17 (chapter 2).

4.2.8 Determination of histone-2AX (H2AX) phosphorylation (γ-H2AX) levels using Immunocytochemistry

The method was based on (Schmid et al., 2010) and previously followed in our lab (Laubenthal et al., 2012). A total of 100 cells per treatment were examined under the fluorescence microscope connected with a CCD camera (Nikon Digital Sight DS-SMC, Surrey, UK) for gamma-H2AX foci expression, a marker

of DNA double strand breaks. Untreated cells were used as the negative control whereas doxorubicin, a known strand-break inducer (50μM) was used as the positive control. Each experiment was done in triplicate with both controls included. (Refer to 2.2.19, chapter 2 for details)

4.2.9 Statistical analysis

Results were presented as mean ± SE and differences between the groups were analysed by one-way analysis of variance (ANOVA) and t-tests using Graph Pad prism 7 software. Values of $p<0.05$ were considered significantly different.

4.3 Results

4.3.1 Preliminary treatment and concentration range study in lymphocytes

Based on the dose response studies 10μM for MYR B and 20μM for MYR N were optimal doses with no cellular toxicity and were used for the experiments throughout the study. H_2O_2 (50μM) was used throughout the study as the positive control and for co-supplementation in the Comet assay, determined by the dose response. TBHP is a known intracellular stress inducer and it was provided with the kit (Abcam, UK) as a positive control therefore we used it to determine the effects of MYR B and MYR N on intracellular ROS levels induced by TBHP. The increase in ROS production was used as a factor to choose the optimum concentration for TBHP i.e. 300μM.

The concentration dependent responses of TBHP are shown in the figure 4.1 below. TBHP has induced significant levels of ROS at each determined concentration after 60mins of treatment. However, at 300μM its activity was at highest when compared to the untreated group.

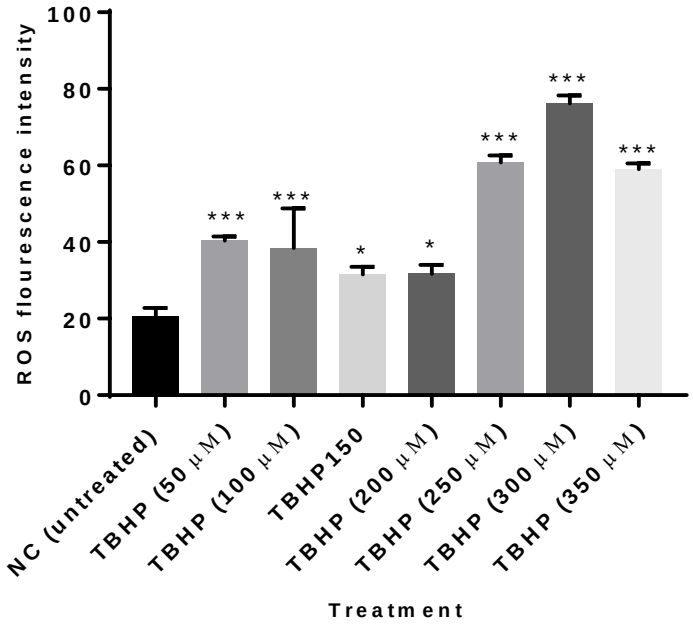


Figure 4.1 Average of 3 independent experiments showing, change in intracellular ROS after treatment with different concentrations of TBHP in healthy lymphocytes. All groups were compared against the untreated group. ***=P<0.001, *P=0.02. Results are expressed as mean ± SD.

4.3.2 Lymphocyte viability

Lymphocytes were treated with different chemicals and their % viability was determined after different time periods counting 100 cells each/ treatment group using the trypan blue exclusion method. Results show that there was no significant difference between the viability of lymphocytes at different time periods and that the concentrations of chemicals used for the study were non-toxic for lymphocytes from healthy individuals.

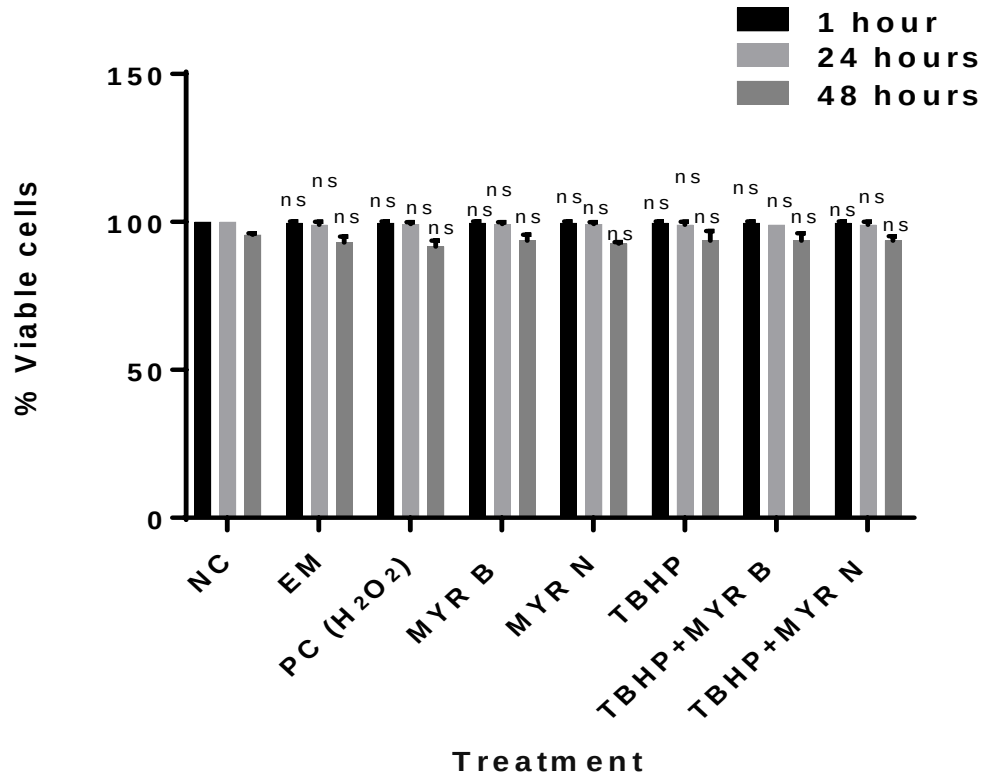


Figure 4.2 % viability per 100 cells counted/treatment of healthy lymphocyte after 1, 24 and 48 hours. Treatment groups used were untreated (NC), excipient mixture (EM), positive control (PC) H_2O_2 50µM, MYR B (10 µM), MYR N (20 µM), TBHP (300µM), TBHP supplemented with MYR B and TBHP supplemented with MYR N. There was no significant difference on viability of cells at different time periods and was calculated more than 80% for all the treatment groups.

4.3.3 Cytotoxicity of chemicals in lymphocytes

Cytotoxicity of test chemicals was determined by culturing lymphocytes from healthy individuals and pre-cancerous patients and measuring the mean absorbance at 1 and 24 hrs using MTT dye. Results from the MTT assay showed that the test chemicals used in this study exhibited significant level of cytotoxicity when compared to the untreated cells. However, % cytotoxicity assessed for each group was lower than 6% after 1 and 24 hours of treatment

in lymphocytes from both healthy and patient group.

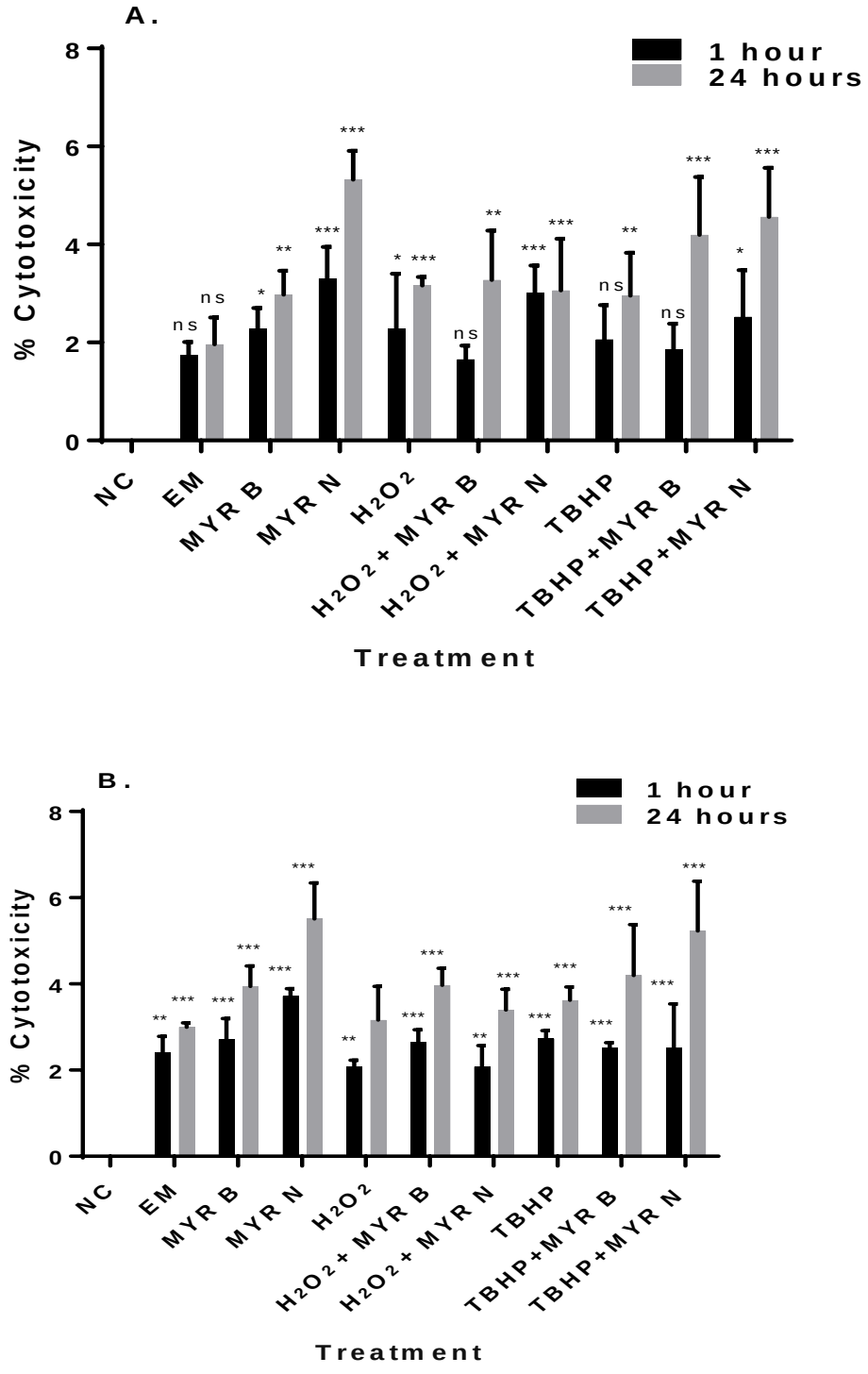


Figure 4.3 % Cytotoxicity in 2x10^4 cells of various test treatments in lymphocytes from healthy volunteers (A) and pre-cancerous patients (B) using MTT assay. Overall values were less than 5% which is not considered as cytotoxic. Error bars show mean values ± SD, *n* = 3. *P=0.03, **P=0.002, ***P=0.0002, ns=not significant.

4.3.4 Effect of MYR N & B on Hydrogen peroxide (H_2O_2) induced DNA damage in lymphocytes from healthy vs patient group using the Comet assay

In-vitro treatment of lymphocytes from healthy individuals and patients with MYR B (10µM) and MYR N (20µM), co-supplemented with 50µM H_2O_2 , resulted in significant reduction of DNA damage when compared to the PC (50µM H_2O_2) alone. PC alone induced significant levels of DNA damage when compared to the NC. MYR N and NC show same level of DNA damage in healthy lymphocytes both in %Tail DNA and OTM. The H_2O_2 induced DNA damage was significantly reduced by MYR B and MYR N in both groups, whereas MYR N was more effective against H_2O_2 induced DNA damage in healthy group when compared to the PC, giving the value almost similar to the untreated group.

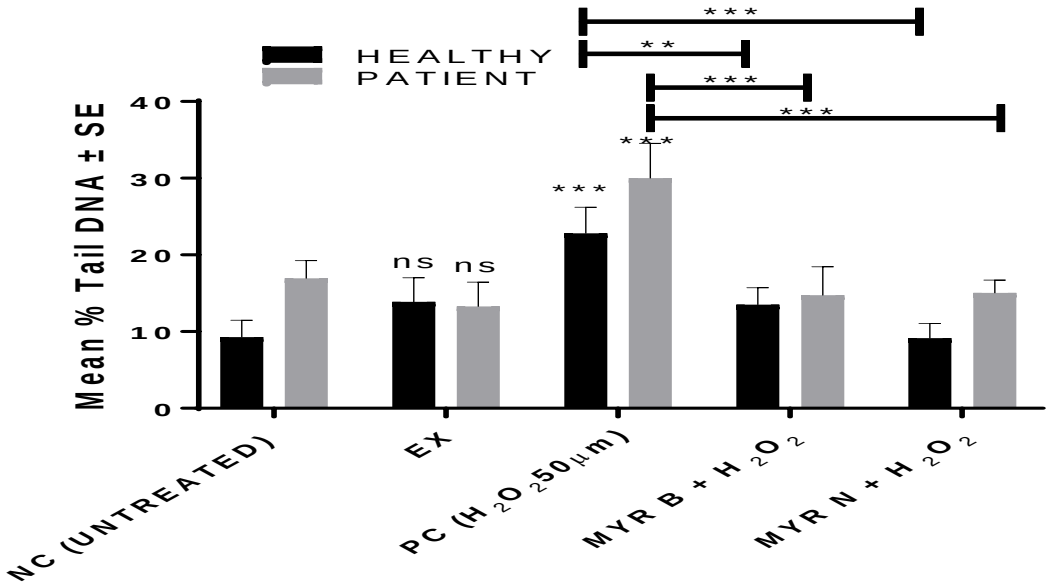


Figure 4.4 (A) Mean % Tail DNA showing effect of MYR N & B on H_2O_2 induced DNA damage in lymphocyte taken from healthy individuals and pre-cancerous patients. Figure shows four groups of treatments including the negative control, excipient mixture, positive control, MYR B (10µM) co-supplemented with H_2O_2 and MYR N (20µM) co-supplemented with H_2O_2. All treatments were compared against the PC. The mean control values for the % tail DNA of NC and PC group for healthy individuals and patients were 9, 22 and 18, 29 respectively, measuring 100 cells each per experiment. * shows significant difference between the groups. The horizontal lines on top of the graph show the significance difference the positive

control and the treatment groups. (For healthy groups ** P< 0.009, *** P< 0.005. For patient groups, *** P< 0.003).

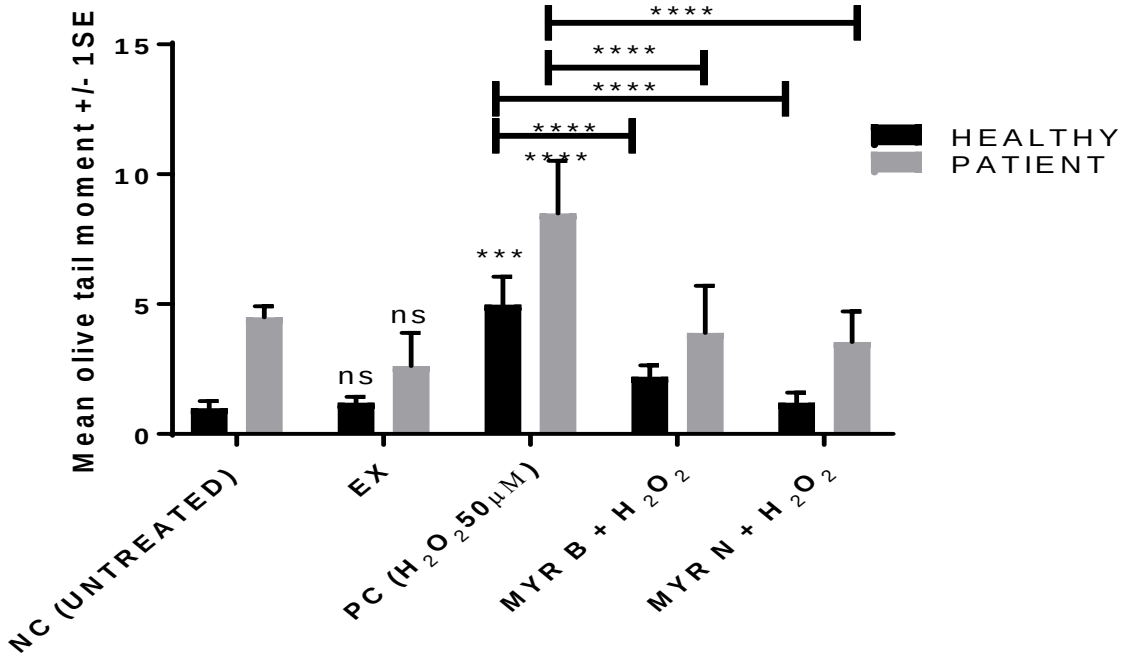


Figure 4.4 (B) Mean OTM showing the effect of MYR B & N on H_2O_2 induced DNA damage in lymphocytes from healthy volunteers and pre-cancerous patients. Figure shows four groups of treatments including the negative control, positive control, MYR B (10µM) co-supplemented with H_2O_2 and MYR N (20µM) co-supplemented with H_2O_2. All treatment groups were compared to the PC group. The mean NC values for the OTM of healthy and patient groups were 0.9 and 4.5 respectively. The mean maximum values of the PC of healthy and patient groups were 5 and 8 respectively. The horizontal lines on top of the graph show the significance difference between the positive control and the treatment groups. (* P< 0.05, ** P< 0.007 shown on healthy groups and ** P< 0.002, **P< 0.002 for patients groups).

4.3.5 Anti-oxidant effects of myricetin

The study used a cell permeant reagent 2',7' –dichlorofluorescin diacetate (DCFDA) that measures total intracellular ROS activity and levels. Once diffused into the cells, cellular esterases deacetylate DCFDA as a non-fluorescent form which is later oxidized to a highly fluorescent compound, 2', 7' –dichlorofluorescein (DCF) by interacting with ROS and easily detected by fluorescence spectroscopy. The effect of MYR B (10µM) and MYR N (20µM) on basal ROS levels was determined in lymphocytes from the healthy group and

pre-cancerous patients. Results showed that MYR N has significantly reduced the ROS levels in lymphocytes from both groups.

4.3.5.1 TBHP-induced oxidative injury in lymphocytes: Protection by myricetin

TBHP, a known stress inducer was used as the PC in this case instead of H_2O_2 because it was provided with the kit.

Exposure of healthy and patient lymphocytes to TBHP alone induced a significant increase in ROS levels by 40%. However, upon treatment with MYR B (10µM) and MYR N (20µM) a significant attenuation of TBHP-induced ROS was observed in lymphocytes from both groups.

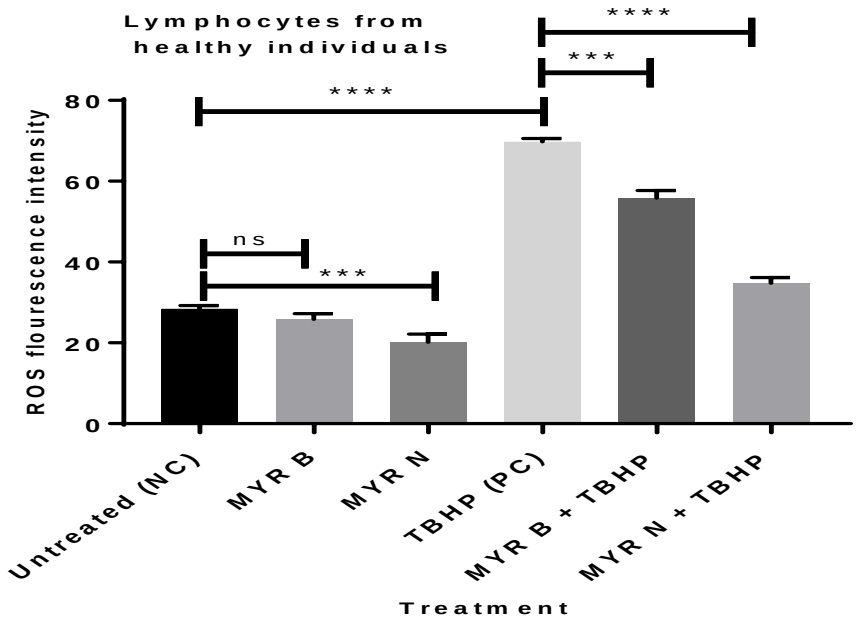


Figure 4.5 Average of 3 independent experiments showing, change in intracellular ROS before and after treatment with TBHP in healthy lymphocyte. The horizontal lines show the significant difference between the groups. ***, ****=P<0.0001, ns=not significant. Six treatment groups included an untreated group (NC), MYR B (10µM), MYR N (20µM), TBHP ((300µM) as PC, TBHP + MYR B and TBHP + MYR N.

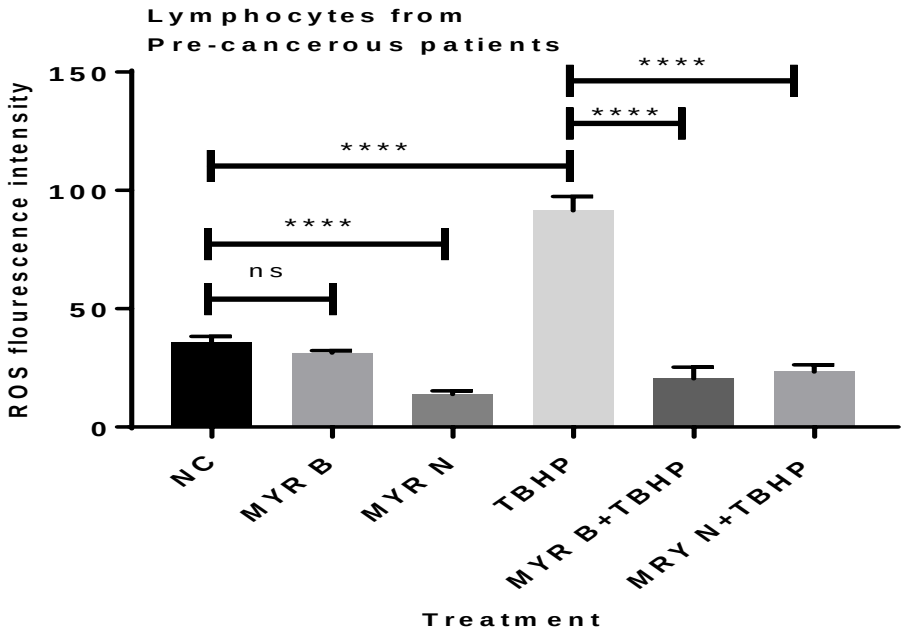


Figure 4.6 Average of 3 independent experiments showing, change in intracellular ROS before and after treatment with TBHP in lymphocyte from pre-cancerous patients. The horizontal lines show the significant difference between the groups. ns = not significant, stars shows P<0.001. Six treatment groups included an untreated group (NC), MYR B (10µM), MYR N (20µM), TBHP ((300µM) as PC, TBHP + MYR B and TBHP+ MYR N.

4.3.6 Activity of intracellular anti-oxidant enzyme, GSH and change in GSH/GSSG ratio

The effects of MYR B and MYR N on the basal levels of GSH were assessed by treating lymphocytes from healthy and patient group with both forms of myricetin alone. MYR B and MYR N have shown a trend to increase the basal levels of GSH/GSSG ratio in lymphocytes from pre-cancerous patients but levels are not significantly different. Hence, myricetin does not show any substantial effect on GSH levels in lymphocytes from healthy individuals and in those from pre-cancerous patients.

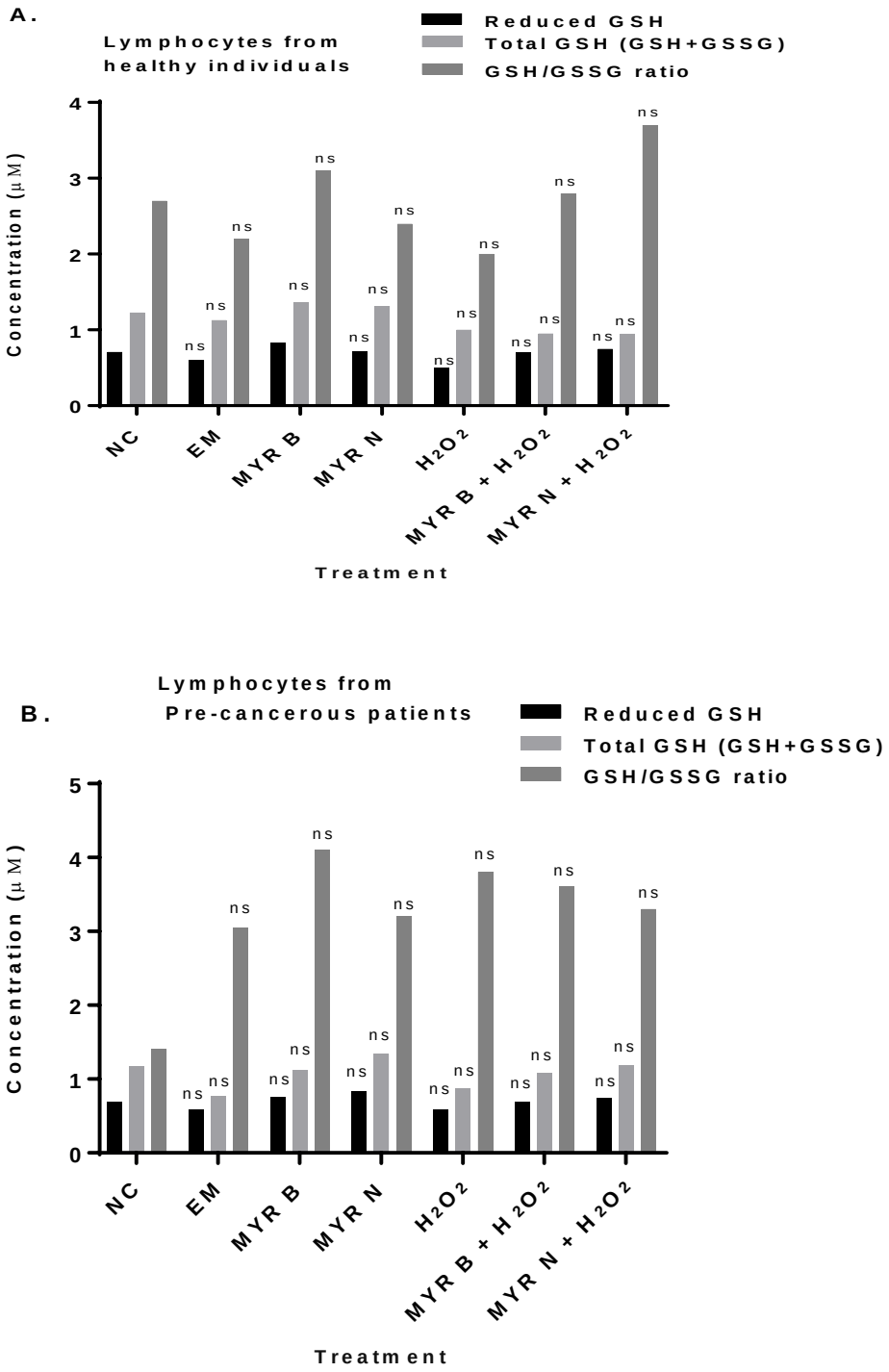


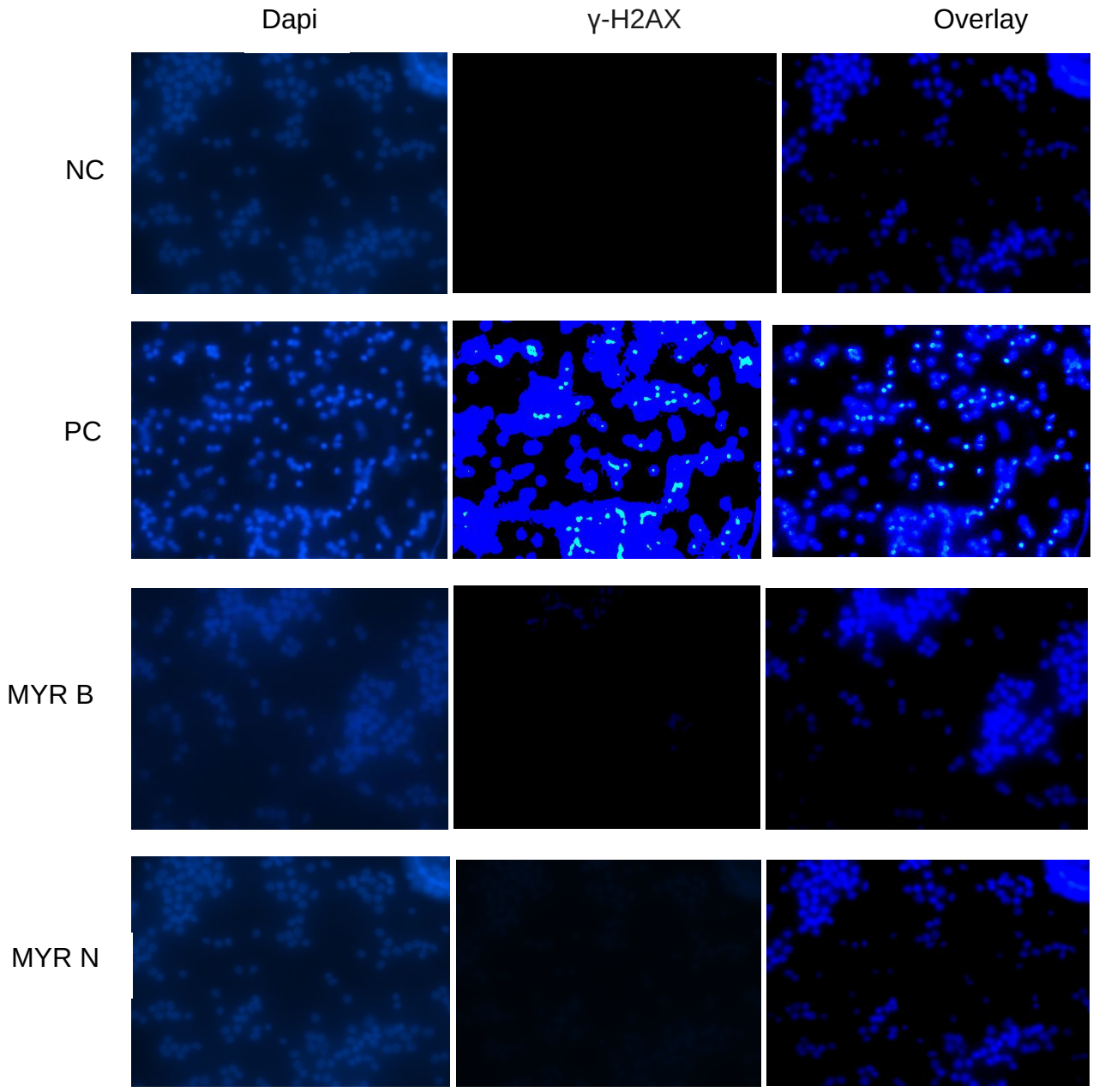
Figure 4.7 Levels of different forms of GSH in healthy lymphocyte (A) and those from pre-cancerous patients (B). Cells lysed to the concentration of 1x 10^5 cells/ml. Various treatment groups included the NC (untreated), EX, MYR B (10µM), MYR N (20µM), H_2O_2 (50µM) as PC , MYR B+H_2O_2 and MYR N+ H_2O_2. The reduced and total GSH increased in healthy lymphocytes as compared to NC and GSH/GSSG ratio also increased by treating healthy lymphocyte with MYR B alone. However, co-supplementation of H_2O_2 with MYR B & MYR N significantly improved the level of reduced GSH and GSH/GSSG ratio when compared to the PC (H_2O_2).

4.3.7 Effect of myricetin on DSB formation in lymphocytes at basal levels

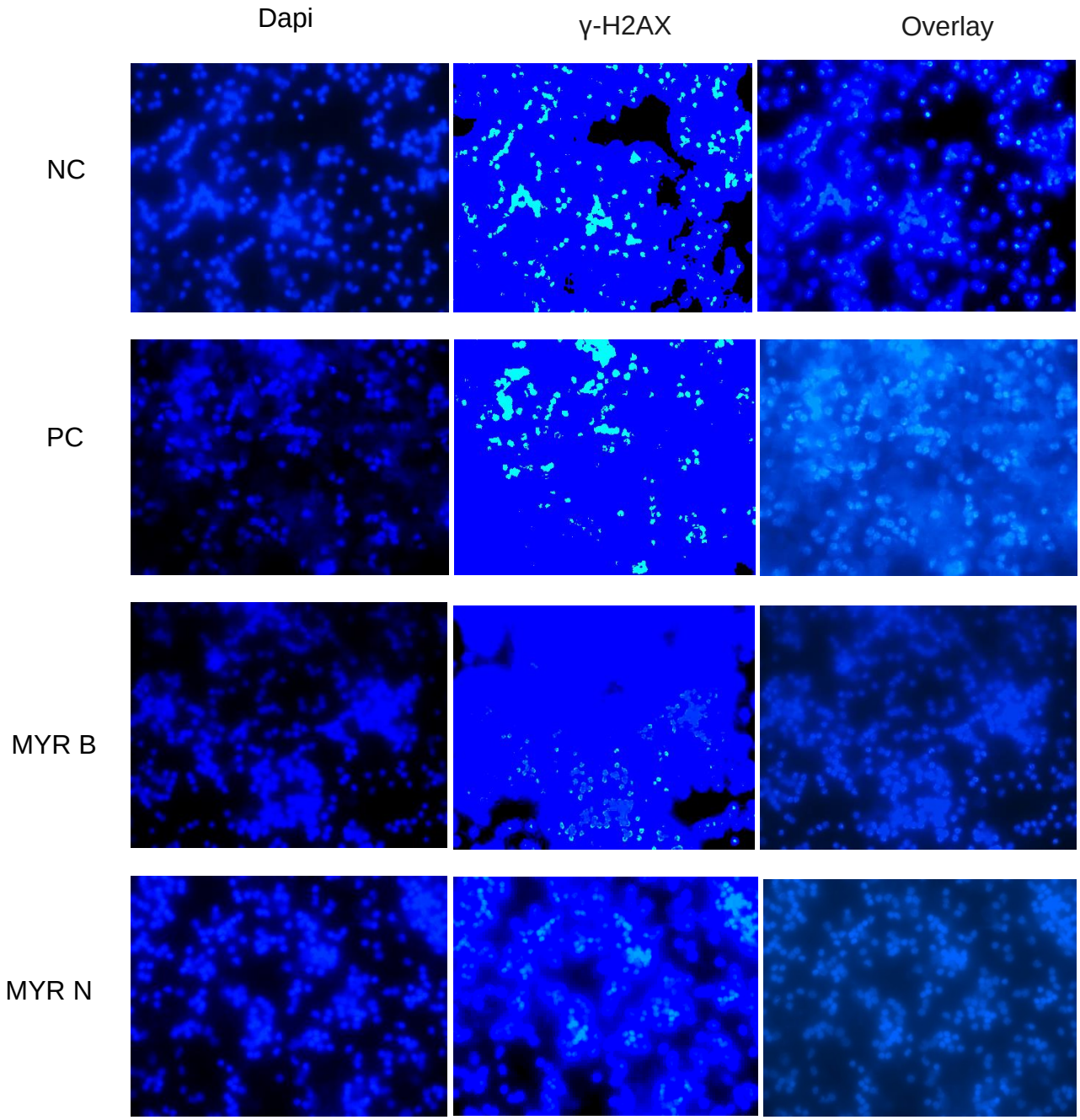
DSBs in the nuclei of lymphocyte cells were immunocytochemically stained using the *γ*H2AX protein and *γ*H2AX foci inside each nucleus were counted in 100 cells each. One DSB represented one *γ*H2AX focus. Results showed that myricetin does not cause DSBs in healthy lymphocytes. Compared to the number of foci generated in the control, both forms of myricetin (MYR B, MYR N) have not shown any significant increase in foci formation. However, as shown in Figure 4.8 B, an increased number of *γ*H2AX foci formation were observed after treatment of the healthy lymphocytes with doxorubicin, a known strand break inducer (PC) ($p<0.001$). Foci formation was observed at basal levels in untreated group from pre-cancerous patients and this incidence of the foci formation was significantly increased ($p<0.001$) after doxorubicin treatments. Upon treatment with MYR B (10μM) and MYR N (20μM) forms in lymphocytes from pre-cancerous patients, no significant effect was observed in *γ*H2AX foci formation compared to the untreated group. This suggests that myricetin does not induce DSBs in healthy lymphocytes at basal levels and could potentially provide protection against DSBs formation in lymphocytes from pre-cancerous patients at basal levels.

Control lymphocytes

A.



Pre-cancerous Patients' lymphocytes



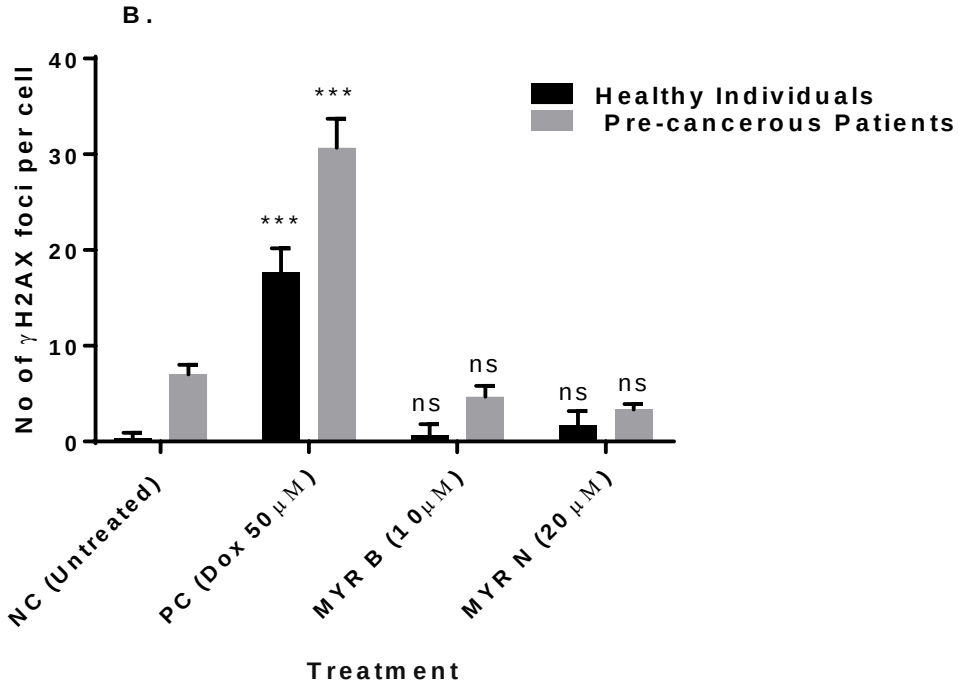


Figure 4.8 *γ*H2AX phosphorylation in lymphocytes from healthy individuals and pre-cancerous patients presented in untreated cells (NC) and treated with doxorubicin (50 µM), MYR B (10 Mm), MYR N (20 µM) at basal levels without any supplementation. (A)Merged Dapi and gamma-H2AX stains in blood lymphocytes from healthy individuals and pre-cancerous patients. Number of phosphorylated H2AX-foci corresponds with DSBs. **(B)** *γ*H2AX foci induction in lymphocytes with different treatment groups. Data were analysed by two-way ANOVA followed by multiple comparison test for significant differences compared to the untreated control for each group (***p < 0.001, ns=not significant).

4.4 Discussion

The Comet assay is a sensitive and reliable method for detection and quantification of DNA damage and DNA strand breaks in single cells. In this study we determined the effects of myricetin against H_2O_2 induced DNA damage by simultaneously exposing the cells to H_2O_2 (50µM) in the presence or absence MYR B or MYR N. The Comet assay results have shown the protective effect of myricetin bulk and nanoparticles (NPs) against H_2O_2 induced ROS related oxidative damage in the lymphocytes of healthy individuals and pre-cancerous patients. The basal DNA damage was significantly inhibited when compared to the positive control (Figure 4.4a, 4.4b). Both forms of myricetin

have shown inhibitory effects whereas MYR N was more effective in causing the reduction in DNA damage, may be due to its enhanced physio-chemical characteristics ($p<0.005$). The potential explanation for this could be that NPs due to their minute size can easily reach the nucleus through diffusion across the nuclear membrane or transportation via the nuclear pores and gain direct interaction with the DNA (Magdolenova et al., 2012; Magdolenova et al., 2014). This could be further investigated and confirmed by studying the sub-cellular distribution of these particles using TEM.

DSBs are most dangerous type of cellular lesions which if left impaired can ultimately lead to cell death. ROS play a crucial role in stimulating DNA strand breaks formation and causing oxidative stress (Tanaka et al., 2007). Accumulation of the DNA damage induced by ROS can lead to various deleterious processes including the stimulation of neoplasms production and eventually tumour development (Tanaka et al., 2007; Vilenchik and Knudson, 2003).To evaluate the anti-oxidant potential of myricetin we investigated its anti-oxidant effects in lymphocytes from healthy individuals and pre-cancerous patients by measuring overall cellular ROS contents. The basal levels of ROS and the effect of myricetin against TBHP-induced ROS were evaluated. Results have demonstrated that myricetin has reduced the basal damage in lymphocytes from both the investigated groups as well as the insults introduced by TBHP treatment (figure 4.5, 4.6) Both forms of myricetin have shown strong anti-oxidant defence by scavenging the free radicals caused by ROS and by reducing their levels intracellularly. However, when two forms were compared, MYR N (20μM) demonstrated more protective effects than MYR B (10μM). Our results are consistent with previous studies (Wang et al., 2010; Kang et al., 2010). Inhibition of ROS may prevent its accumulation, also the interaction of

floating radicals with cellular contents and DNA fragmentation, avoiding DNA lesions and strand breaks. Hence, myricetin shows anti-mutagenic and anti-carcinogenic properties by preventing mutations at non-genotoxic concentrations used for both forms of myricetin. These results are consistent with previous studies conducted on various cells lines using myricetin. It has been previously shown that myricetin protect cells from H_2O_2 damage by inhibiting ROS production and by stimulating the antioxidant enzymes. It restored the function of antioxidant defence enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) which was reduced by H_2O_2 treatment (Wang et al., 2010).

Although we used the fluorescent probe DCFDA to detect ROS levels, the formamido pyrimidine glycosylase (FPG) protein could also be considered to assess oxidative DNA damage using the Comet assay (Müller, et al., 2013).

Furthermore, this study also investigated the effect of myricetin on intracellular GSH levels by using a highly sensitive proprietary non-fluorescent dye which becomes fluorescent when reacted with GSH. Glutathione occurs in two forms; reduced (GSH) and oxidized form, glutathione disulphide (GSSG). Reduced glutathione (GSH) is a key tissue antioxidant and presents first line of defense against ROS. Reduced Glutathione (GSH) is the smallest intracellular protein thiol molecule present in the cells that inhibits ROS (such as free radicals and peroxides) induced cell damage. Oxidized glutathione (GSSG) is produced by the formation of a disulfide bond between two GSH molecules in the glutathione peroxidase (GPx) catalyzed reaction. The enzyme glutathione reductase (GR) reprocesses GSSG to GSH. In healthy cells, the total glutathione pool is mostly in the reduced form (GSH). However, when cells are exposed to increased

levels of oxidative stress, GSSG starts gathering and the ratio of GSSG to GSH rises. Hence, a bigger ratio of GSSG-to-GSH is an indication of oxidative stress (Roy and Sil, 2012).

Our results have demonstrated no significant effect on GSH levels after treatment with MYR B and MYR N in lymphocytes from healthy and patient groups (figure 4.7).

Gamma-H2AX is used as an effective biomarker of DSBs. An accumulating body of evidence suggests that the crucial role of γH2AX phosphorylation for nuclear foci formation at DBS sites and stimulation of DNA repair (Rogakou et al., 1998; Podhorecka et al., 2010). To evaluate the effect of myricetin on DSBs formation in lymphocytes from healthy individuals and those from pre-cancerous patients we quantified γH2AX foci intensity using immunofluorescence. Our results have shown that myricetin does not induce DSBs formation in healthy lymphocyte and those from the pre-cancerous patients at basal levels which is consistent with our previous results from the Comet assay where no significant DNA damage was caused upon exposure to MYR B (10μM) and MYR N (20μM)(figure 4.8). However, a trend to lower an overall intensity of γH2AX foci was observed in the lymphocytes from pre-cancerous patients treated with MYR B and MYR N when compared to the untreated group. This marks the current study as the first one to demonstrate the effect of MYR B (10μM) and MYR N (20μM) on DSBs development in lymphocytes from healthy individuals and those from pre-cancerous patients at basal levels.

In conclusion, this work demonstrates for the first time (to our knowledge) that myricetin bulk and nano at selective non-genotoxic concentrations protect the lymphocytes (from healthy individuals and pre-cancerous patients) from

damaging effects of TBHP and H_2O_2 primarily by inhibiting ROS-induced oxidative stress. Besides this, MYR B (10μM) and MYR N (20μM) do not induce formation of DSBs in lymphocytes from healthy individuals and pre-cancerous patients at basal levels and could possibly protect the lymphocytes from extensive cell damage by inhibiting DSBs formation and ultimately help in cell survival and DNA damage repair. DNA repair capacity of lymphocytes after treatment with myricetin could possibly studied using the Comet assay simply by assessing the damage at different time periods (Collins, 2004) The overall results have demonstrated that MYR N (20μM) has shown better antioxidant and genoprotective effects against the oxidative damage in lymphocytes from healthy individuals and pre-cancerous patients when compared to MYR B (10μM).

Chapter 5: The protective effect of myricetin bulk and nanoparticles on PhIP (2- amino-1-methyl-6 phenylimidazo [4,5-b] pyridine)-induced DNA damage in lymphocytes from healthy individuals and pre-cancerous patients

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5.1 Introduction

Recently, the consumption of processed and overcooked red meat has been associated with causing carcinogenicity in humans (Bouvard et al., 2015), attributed to the production of food-related carcinogens including heterocyclic amines (HCAs) (Sugimura et al., 2004). HCAs are strong DNA-damaging complexes which are formed when meat and other related products are cooked at very high temperature (Turesky and Le Marchand, 2011). 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP) is considered as most commonly occurring HCA in our diet (Sugimura et al., 2004). It is metabolically activated by cytochrome P450 1A2 (CYP1A2) producing toxic intermediate, 2-hydroxyamino-1-methyl-6- phenylimidazo [4,5-*b*]pyridine (N-OH-PhIP), followed by additional sulfonation by sulfotransferases (SULTs) or acetylation by N-acetyl-transferases (NATs) (Turesky, 2007). It has been suggested that these compounds reach the distal colon through the bloodstream and are actively released into the lumen (Nicken et al., 2013). C8-PhIP-dG adducts, bulky DNA lesions are formed due to the instability of these esters by releasing a DNA-reactive arylnitrenium ion. These adducts undergo nuclear excision repair (NER) to fix the damage. Unrepaired C8-PhIP-dG adducts primarily cause single nucleotide deletions and G:T conversion mutation, hence are pre-mutagenic (Morgenthaler and Holzhauser, 1995; Yadollahi et al., 1996)). It has been stated that increasing PhIP doses cause cellular death while surviving cells exhibit high levels of mutations, determined in the hypoxanthine-guanine phosphoribosyl transferase *(hprt)* locus (Gooderham et al., 2002). Several *in vivo* (Cheung et al., 2011; Choudhary et al., 2012; Li et al., 2012) and epidemiological studies (Cross et al., 2005; Voutsinas et al., 2013) reported the contribution of PhIP towards the induction of mammary, gastrointestinal and prostate cancers in rodents. C8-

PhIP-dG adducts are said to interfere with the DNA replication process triggering a cellular stress response called replication stress mainly through DNA damage which ultimately gives rise to single stranded DNA (ssDNA) **(**Byun et al., 2005). The ssDNA quickly forms ssDNA-RPA complex by adjoining with the replication protein A (RPA) which are then detected by ATR-interacting protein (ATRIP) recruiting kinase ATR. Thus, this process triggers DNA damage response (DDR) by activating ATR (Zou and Elledges, 2003; Ball et al., 2005). ATR plays a significant role in phosphorylation of various downstream effectors such as the checkpoint kinase CHK1, histone H2A (H2AX) and the cell cycle protein, RAD17 (Zeman and Cimprich, 2014). ATR in cooperation with RPA, therefore, induces the repair pathways and facilitates the restart of hindered replication forks by stabilizing them (Cimprich and Cortez, 2008). Continuous replication stress leads to the formation of DNA double strand breaks (DSBs) (Zeman and Cimprich, 2014) and recruitment of kinase ATM (Shiloh and Ziv, 2013).

Myricetin, a well-studied flavonoid with diverse properties is primarily recognised due to its anti-oxidant, anti-cancer and anti-inflammatory activities (Ong and Khoo, 1997). Research studies present no doubt for the compound to be a strong anti-oxidant. It has also been regarded as a potent chemo preventative agent against various tumours (Androutsopoulos et al., 2011; Kim et al., 2014). Myricetin displayed anti-genotoxic effects against the food mutagens, 3-amino-1-methyl -5H-pyrido-(4,3-b) indole (Trp) and 2-amino-3-methylimidazo-(4,5-f) quinoline (IQ) and decreased DNA damage without exogenous metabolic initiation in human lymphocyte cells (Anderson et al., 1997). However, on the other hand the compound triggered the basolateral

uptake of PhIP through partly preventing the MRP2-regulated excretion of PhIP from intestine back to the lumen (Schutte et al., 2006).

Diet is an important contributory factor towards the development of various cancers and knowing that lymphocytes express CYP1A2 and that the food mutagen, PhIP, activated by CYP1A2, contributes in many dietary tumours (Anderson, et al., 1997; Cheung et al., 2011; Voutsinas et al., 2013), we investigated for the first time in this study the effects of PhIP on basal levels as well as by co-supplementation with either MYR B or MYR N in lymphocytes from healthy individuals and pre-cancerous patients. This was conducted to determine if PhIP causes DNA damage in lymphocytes and whether dietary compounds like myricetin could help to reduce the harmful effects of PhIP and prevent cancer development in pre-cancerous patients.

In this study we investigate and analyse the effects of PhIP treatment on the induction of DNA damage, strand breaks formation, intracellular glutathione (GSH) levels, kinase ATR regulation, p53 and Bcl-2 levels in peripheral lymphocytes from pre-cancerous patients and healthy individuals and also the modulating effects of myricetin (MYR B and MYR N) on PhIP-induced metabolic changes of these factors. Using the Comet and micronucleus assays we investigated the effect of PhIP treatment in lymphocytes from healthy individuals and pre-cancerous patients and the modulating effects of myricetin. We also determined the anti-oxidant potential of myricetin in human lymphocytes against PhIP-induced oxidative stress by analysing the levels of intracellular anti-oxidant enzyme, GSH. Using western blotting and real-time PCR we studied the influence of chemicals on ATR, P53 and Bcl-2 pathways at m RNA and protein levels in isolated lymphocytes.

5.2 Methodology

For cell culture, reagents, cell viability, the Comet assay procedure, micronucleus assay, assay of cellular enzyme and total thiol content, Western blot analysis and the real-time RT-PCR analysis please refer to chapter 2 for more details. The blood samples from healthy individuals and pre-cancerous patients used for this study are listed in table 5.1 and 5.2 below.

No	Age	Ethnicity	Gender	Smoking history	Family history
1	25	CAUCASIAN	M	NO	NONE
2	44	ASIAN	M	YES	NONE
3	28	CAUCASIAN	M	NO	NONE
4	23	CAUCASIAN	F	NO	NONE
5	27	CAUCASIAN	M	NO	KIDNEY CANCER
6	33	ARAB	M	YES	NONE
7	47	ASIAN	M	YES	NONE
8	28	CAUCASIAN	M	NO	NONE
9	42	ASIAN	M	NO	NONE
10	48	ASIAN	M	NO	NONE
11	60	ASIAN	M	YES	NONE
12	24	ASIAN	M	NO	NONE
13	34	ASIAN	M	NO	NONE
14	34	CAUCASIAN	F	YES	NONE
15	34	ASAIN	M	NO	NONE
16	59	CAUCASIAN	F	YES	NONE
17	28	ASIAN	M	YES	NONE
18	61	CAUCASIAN	F	NO	NONE
19	36	CAUCASIAN	F	NO	NONE
20	52	CAUCASAIN	F	NO	NONE

Table 5.1 Brief information on healthy blood samples used in this chapter

No	Age	Ethnicity	Gender	Smoking history	Family history	Medical condition
1	63	CAUCASIAN	M	YES	NONE	MGUS
2	75	CAUCASIAN	M	NO	NONE	MGUS
3	74	CAUCASIAN	F	NO	LUNG CANCER	MGUS
4	83	CAUCASIAN	M	NO	NONE	MGUS
5	60	ASIAN	F	NO	NONE	MGUS
6	62	CAUCASIAN	M	NO	NONE	MGUS
7	51	CAUCASIAN	F	NO	NONE	MGUS
8	80	CAUCASIAN	M	NO	CANCER POSITIVE	MGUS
9	81	CAUCASIAN	F	NO	BOWEL& STOMACH	MGUS
10	63	CAUCASIAN	M	YES	NONE	MGUS
11	63	CAUCASIAN	M	YES	NONE	MGUS
12	74	CAUCASIAN	M	NO	NONE	MGUS COPD
13	63	CAUCASIAN	F	YES	ARTHRITIS	MGUS, COPD
14	66	CAUCASIAN	F	NO	BREAST CANCER	MGUS
15	52	CAUCASIAN	M	YES	NONE	MGUS
16	79	CAUCASIAN	M	NO	NONE	MGUS, COPD, MONOCLONAL B CELL LYMPHOCYTOSIS
17	80	CAUCASIAN	F	NO	NONE-	MGUS
18	78	CAUCASIAN	M	NO	NONE-	MGUS
19	50	ASIAN	F	NO	NONE	MGUS
20	69	CAUCASIAN	M	NO	STOMACH AND LUNG	MGUS

Table 5.2 Brief information of pre-cancerous patients' blood samples used in this chapter

5.2.1Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). Graph Pad prism 7 was used to perform statistical calculation. The results were analysed using t-tests, one-way analysis of variance (ANOVA) and two-way ANOVA to test differences between each treatment and control. A p-value of <0.05 was considered statistically significant.

5.3 Results

5.3.1 Viability of lymphocytes

There was no significant effect observed on viability of lymphocytes from healthy individuals and those from pre-cancerous patients after 24 hours treatment with various treatment groups used in this study except PhIP + MYR N. However, viability for this group was also assessed more than 86% in both healthy and patient lymphocytes (figure 5.1). This suggests that the concentration of chemicals used throughout the study were non-toxic for the lymphocyte cells.

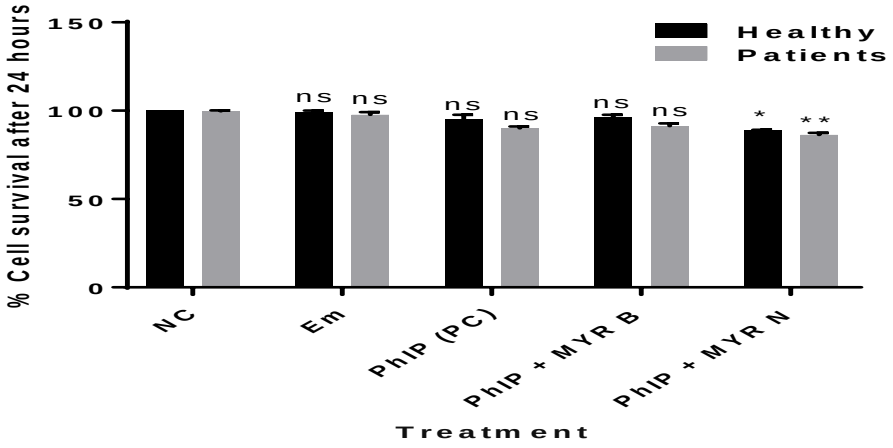


Figure 5.1 % viability per 100 cells counted/treatment after 24 hours. Treatments groups used were untreated (NC), excipient mixture (EM), positive control (PC) PhIP 100µM, PhIP supplemented with MYR B (10µM) and MYR N (20µM). Viability was calculated as more than 80% for all the treatment groups. (ns=not significant, *P<0.02, **P<0.004)

5.3.2 Dose response curve for PhIP and Myricetin

The optimal dose of PhIP inducing maximum DNA damage was determined using the Comet assay in lymphocytes from healthy vs patient group. PhIP concentrations (50-200μM) were considered for the test by comparing against the untreated group. 50μM H_2O_2 was used as relative positive control. Results demonstrate that all doses of PhIP have induced significant ($p<0.001$) DNA damage in healthy lymphocytes and insignificant damage in patients lymphocytes (figure 5.2). Although there was little difference between the three concentrations (50, 100 and 200 μM) I decided to use 100μM as a standard which caused maximum genotoxicity in both groups. Hence, it was used throughout the study.

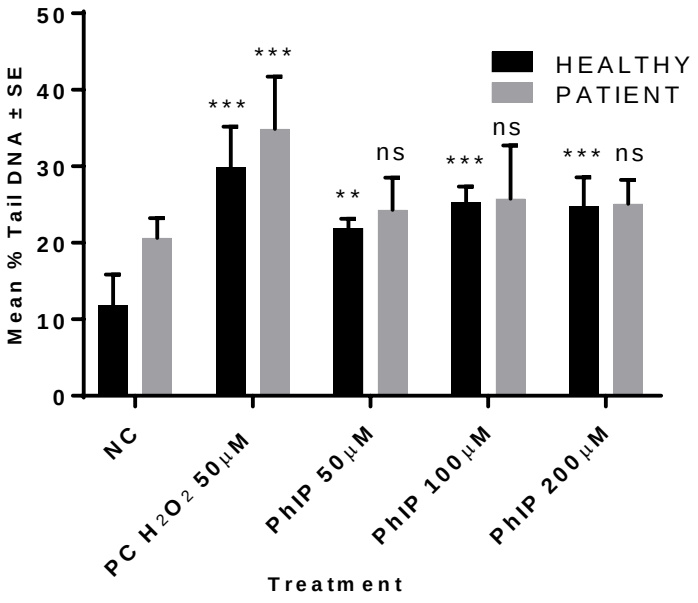


Figure 5.2 Concentration dependent responses of PhIP in lymphocytes from healthy individuals and pre-cancerous patient lymphocyte showing mean %Tail DNA. All PhIP doses induced genotoxic damage to the cells whereas 100μM seemed to produce maximum DNA damage in both groups. All data have been expressed as mean ± standard errors (SE). ***p<0.001, **p<0.01, ns=not significant

The optimal doses for MYR B and MYR N, 10µM and 20µM respectively were determined using the dose response curve and used throughout in the current study as well. The data have already been shown in chapter 3.

5.3.3 Modulating effects of MYR B and MYR N on PhIP-induced DNA damage in lymphocytes using the Comet assay

To determine the *in vitro* effects of different particle sizes of myricetin on PhIP-induced DNA damage, lymphocytes from healthy volunteers and pre-cancerous patients were treated with MYR B (10µM) and MYR N (20µM) simultaneously co-supplemented with PhIP (100µM). Results demonstrate a reduction in DNA damage overall, when compared against the PC (Phip 100µM). The damage was significantly decreased by both forms of myricetin in healthy lymphocytes as well those from pre-cancerous patients (Figure 5.3a,b and 5.4a,b) assessed using the two parameters of the Comet assay, Olive tail moment (OTM) and % Tail DNA. The levels were almost returning to those similar to the negative control. This indicates that MYR B and MYR N exhibit similar protective and anti-genotoxic effects and can potentially protect the lymphocytes of healthy individuals and pre-cancerous patients against the DNA damage and genotoxicity caused by PhIP.

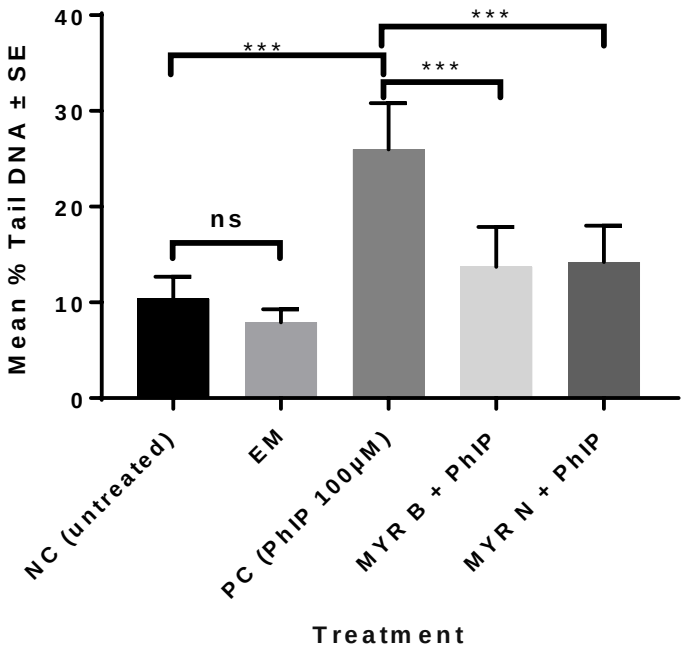


Figure 5.3a Modulating effect of MYR B & N on PhIP induced DNA damage in healthy lymphocytes using % Tail DNA The above figure shows five treatment groups including an untreated group, the positive control (PhIP 100µM), MYR B (10µM)with PhIP, MYR N (20µM) supplemented with PhIP and excipient mixture (EM)(0.1 %). The PC and EM were compared against the NC while MYR B and MYR N against the PC. The mean NC and PC values for healthy groups were 10 and 25 respectively. *** represents P< 0.001, ns = not significant. The horizontal lines on top of the graph show the significant difference between the treatment groups.

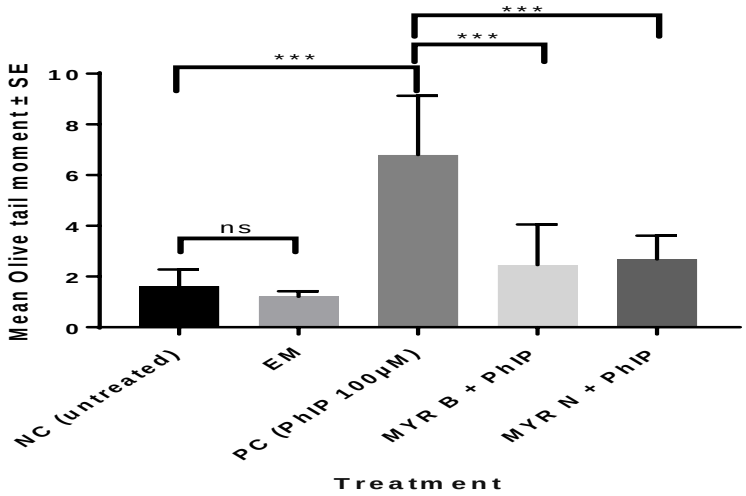


Figure 5.3b Modulating effect of MYR B & N on PhIP induced DNA damage in healthy lymphocytes using Olive Tail Moment The above figure shows five treatment groups including an untreated group, the positive control (PhIP 100µM), MYR B (10µM)with PhIP, MYR N (20µM) supplemented with PhIP and excipient mixture (EM)(0.1 %). The PC and EM were compared against the NC while MYR B and MYR N against the PC. The mean NC and PC values for healthy groups were 1 and 6 respectively. *** represents P< 0.001, ns = not significant. The horizontal lines on top of the graph show the significant difference between the treatment groups.

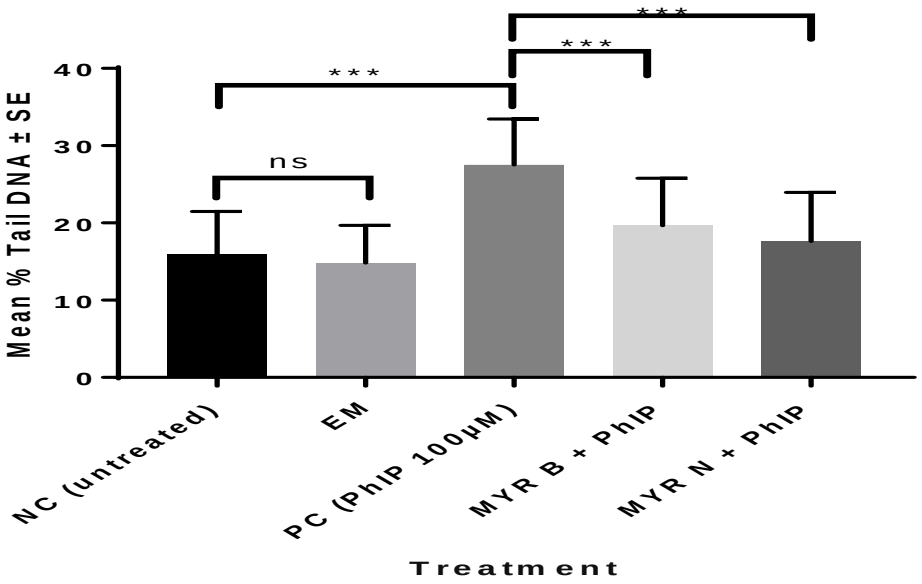


Figure 5.4a Modulating effect of MYR B & N on PhIP induced DNA damage in pre-cancerous patient lymphocytes using % Tail DNA The above figure shows five treatment groups including an untreated group, the positive control (PhIP 100µM), MYR B (10µM)with PhIP, MYR N (20µM) supplemented with PhIP and excipient mixture (EM)(0.1 %). The PC and EM were compared against the NC while MYR B and MYR N against the PC. The mean NC and PC values for healthy groups were 15 and 27 respectively. ***, *** represents P< 0.001, P< 0.002 ns = not significant. The horizontal lines on top of the graph show the significant difference between the treatment groups.

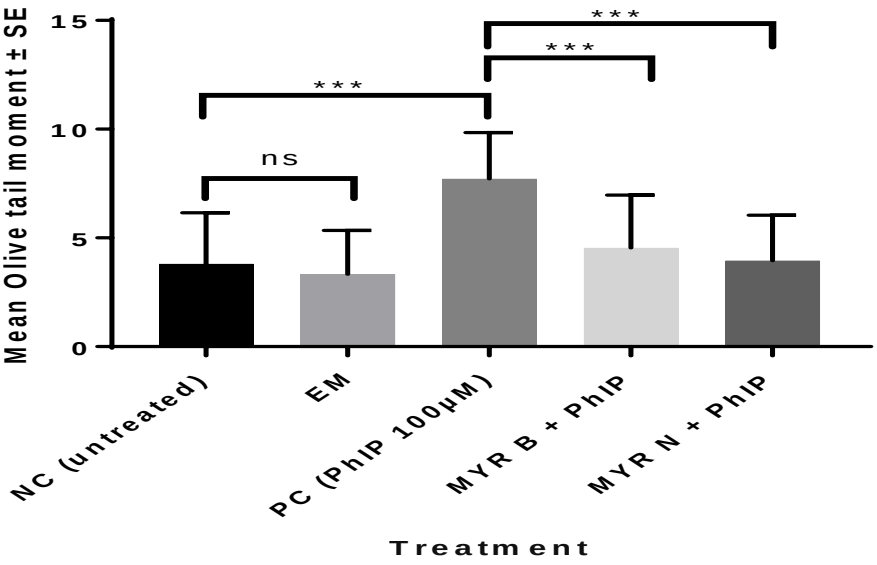


Figure 5.4b Modulating effect of MYR B & N on PhIP induced DNA damage in pre-cancerous patient lymphocytes using Olive Tail Moment The above figure shows five treatment groups including an untreated group, the positive control (PhIP 100µM), MYR B (10µM)with PhIP, MYR N (20µM) supplemented with PhIP and excipient mixture (EM)(0.1 %). The PC and EM were compared against the NC while MYR B and MYR N against the PC. The mean NC and PC values for healthy groups were 3 and 7 respectively. ***P< 0.001, ns = not significant. The horizontal lines on top of the graph show the significant difference between the treatment groups.

5.3.4 Determination of micronuclei (MNi) and other DNA damage

5.3.4.1 MNi frequency in binucleated cells (BiNC)

The effect of PhIP alone and combination of PhIP with MYR B and MYR N on micronuclei formation was assessed using the micronucleus assay. Our results show that the number of MNi in BiNC cells from pre-cancerous patients was higher than those from healthy individuals in their respective untreated groups.There were few MNi observed in the healthy NC group per 1000 cells counted, and this needs to be taken into consideration when evaluating these results. This frequency was further enhanced by exposing the cells to PhIP (100μM) ($p<0.001$). However, complementation of MYR B and MYR N has significantly reduced MNi formation induced by PhIP in BiNC cells from healthy individuals and pre-cancerous patients (figure 5.5). This has been shown on graph using the statistics under the horizontal bars where two supplemented groups were compared against the PC group (PhIP).

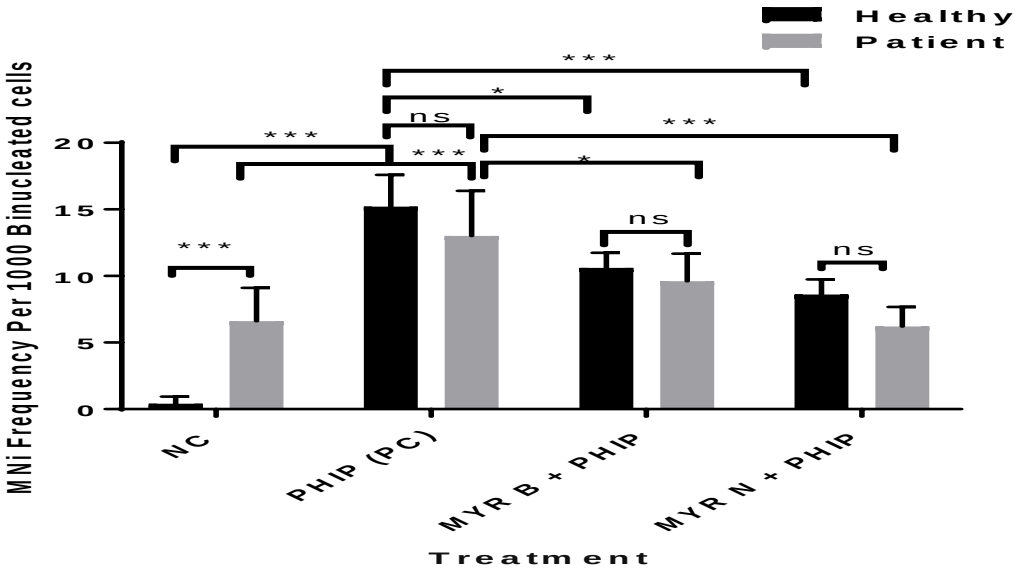


Figure 5.5 Shows the average of BiNC MNi scored per 1000 cells per culture from 5 independent experiments, n=1000. Data are expressed as means ± standard errors (SE). Four treatment groups included the negative control (NC), a positive control PhIP (PC) (100μM) MYR B (10μM) and MYR N (20μM) groups supplemented with PhIP (100μM). (*represents P<

0.01, *** represents P< 0.001). Horizontal lines and the statistics above them show the comparison between the groups.

5.3.4.2 Other elements of MN

Table 5.3 below shows that there is no significant difference between the NDI and % of BiNC for all treatment groups when compared to the respective untreated group for healthy individuals and pre-cancerous patients. PhIP significantly induced MNi formation in both groups. Untreated cultures of patient group have shown higher number of MNi both in MoNC and BiNC as compared to the groups treated with MYR B and MYR N. MYR B (10μM) or MYR N (20μM) addition with PhIP has significantly reduced the MNi induction regardless of group difference and cell type.

Subject	Treatment Group	NDI	% BiNC	MNi in MoNC	MNi in BiNC
Healthy individuals	**Untreated lymphocytes (NC)**	1.85	62	0	0
	PhIP (PC)	1.63 (ns)	62 (ns)	7 (P<0.001)	15 (P<0.001)
	MYR B + PhIP	1.83 (ns)	61 (ns)	4 (P<0.01)	10 (P<0.01)
	MYR N + PhIP	1.63 (ns)	61 (ns)	3 (P<0.01)	8 (P<0.001)
Pre-cancerous patients	**Untreated lymphocytes**	1.81	61	6	6
	PhIP (PC)	1.78 (ns)	61 (ns)	12 (P<0.001)	13 (P<0.001)
	MYR B + PhIP	1.73 (ns)	60 (ns)	8 (P<0.01)	9 (P<0.01)
	MYR N + PhIP	1.80 (ns)	60 (ns)	7 (P<0.01)	6 (P<0.001)

Table 5.3 The average of various markers/parameters of chromosomal damage in the cytokinesis block micronucleus assay. Showing NDI per treatments on healthy and patient cells (all values compared against respective untreated group), mean % of BiNC (all values compared against respective untreated group), mean number of MNI in BiNC and MNi frequency in MoNC (For these two columns, the PC is compared against the untreated lymphocytes for both investigative groups and co-supplemented groups were compared against the PC).

5.3.5 Activity of intracellular anti-oxidant enzyme, GSH and change in GSH/GSSG ratio

This test was carried out by using a highly sensitive proprietary non-fluorescent dye which becomes fluorescent when reacted with GSH. *In-vitro* treatment of lymphocytes from healthy individuals and patients with mutagen PhIP (100µM) alone and co-supplementation with MYR B (10µM) and MYR N (20µM) has shown non-significant effect on GSH levels or GSH/GSSG ratio.

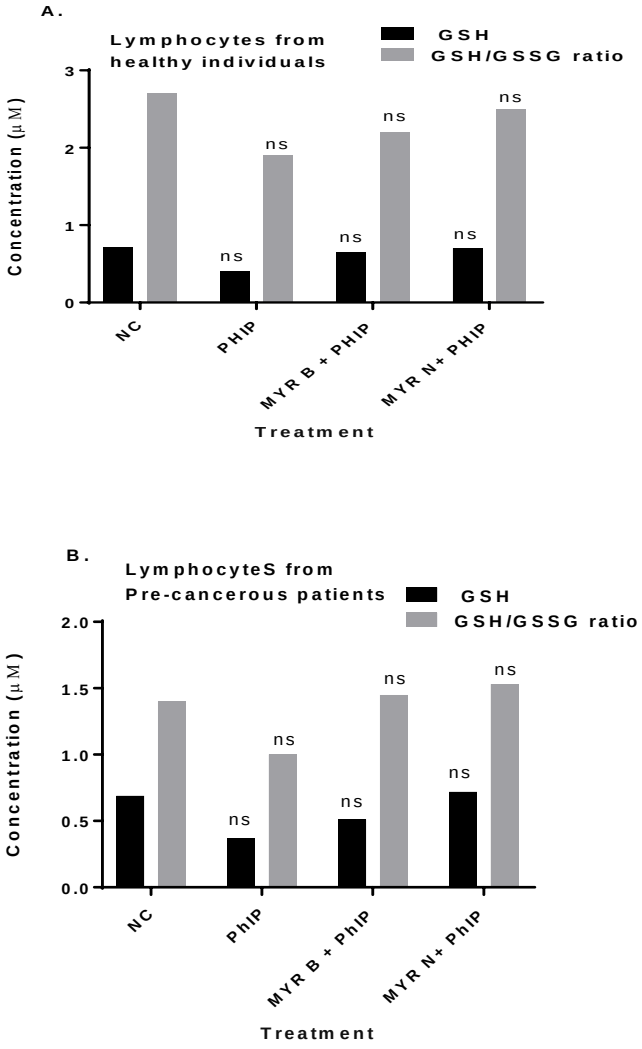


Figure 5.6 Effect of phip on GSH levels and modulation of phip-induced oxidative stress by MYR B and MYR N in healthy lymphocyte (A) and those from pre-cancerous patients (B). Cells lysed to the concentration of 1x 10^5 cells/ml. Various treatment groups included the NC (untreated), PhIP (100µM) as PC, MYR B (10µM)+PhIP and MYR N (20µM)+ PhIP.

5.3.6 Activation the P53 and ATR signalling pathway by myricetin bulk and nanoparticles following PhIP-induced oxidative stress

Built on earlier results from the Comet and micronucleus assays we found that MYR B and MYR N have shown protective effects against PhIP induced DNA damage in lymphocytes from healthy individuals and pre-cancerous patients. To identify the molecular mechanism involved in this effect, we studied the influences of PhIP and then myricetin co-supplementation with PhIP on the gene expression levels of P53, a tumour-suppressor multi-functional gene and ATR kinase mRNA in lymphocytes. Total RNA was isolated from lymphocytes pre-treated with chemicals for 24 hours and subjected to quantitative real-time PCR analysis. The results (Figure 5.7) have shown that in healthy lymphocytes, PhIP treatment significantly decreased the P53 gene expression to 0.5-fold, however, this was significantly up-regulated upon supplementation with MYR B to 1.4-fold and with MYR N to an 1.75-fold increase. In lymphocytes from the patient group the P53 was slightly down-regulated with PhIP treatment whereas significantly up-regulated by MYR N co-supplementation ($p<0.01$). PhIP has shown different effects on gene expression levels of ATR in lymphocytes from healthy individuals to those from pre-cancerous patients. The ATR gene was significantly ($p<0.001$) up-regulated by PhIP in healthy lymphocytes and it was further enhanced by myricetin supplementation where MYR N has shown $p<0.001$ significance. However, PhIP has demonstrated reverse effects on ATR expression in lymphocytes from the patient group. It significantly down-regulated the ATR gene levels ($p<0.01$) which were significantly up-regulated by myricetin addition. These results indicate that protective effects caused by myricetin on PhIP-induced damage might be dependent on the tumour-suppression activity of P53 gene. The mechanisms involved in diverse ATR

regulation by PhIP in lymphocyte from healthy individuals compared to those from pre-cancerous patients are not fully understood.

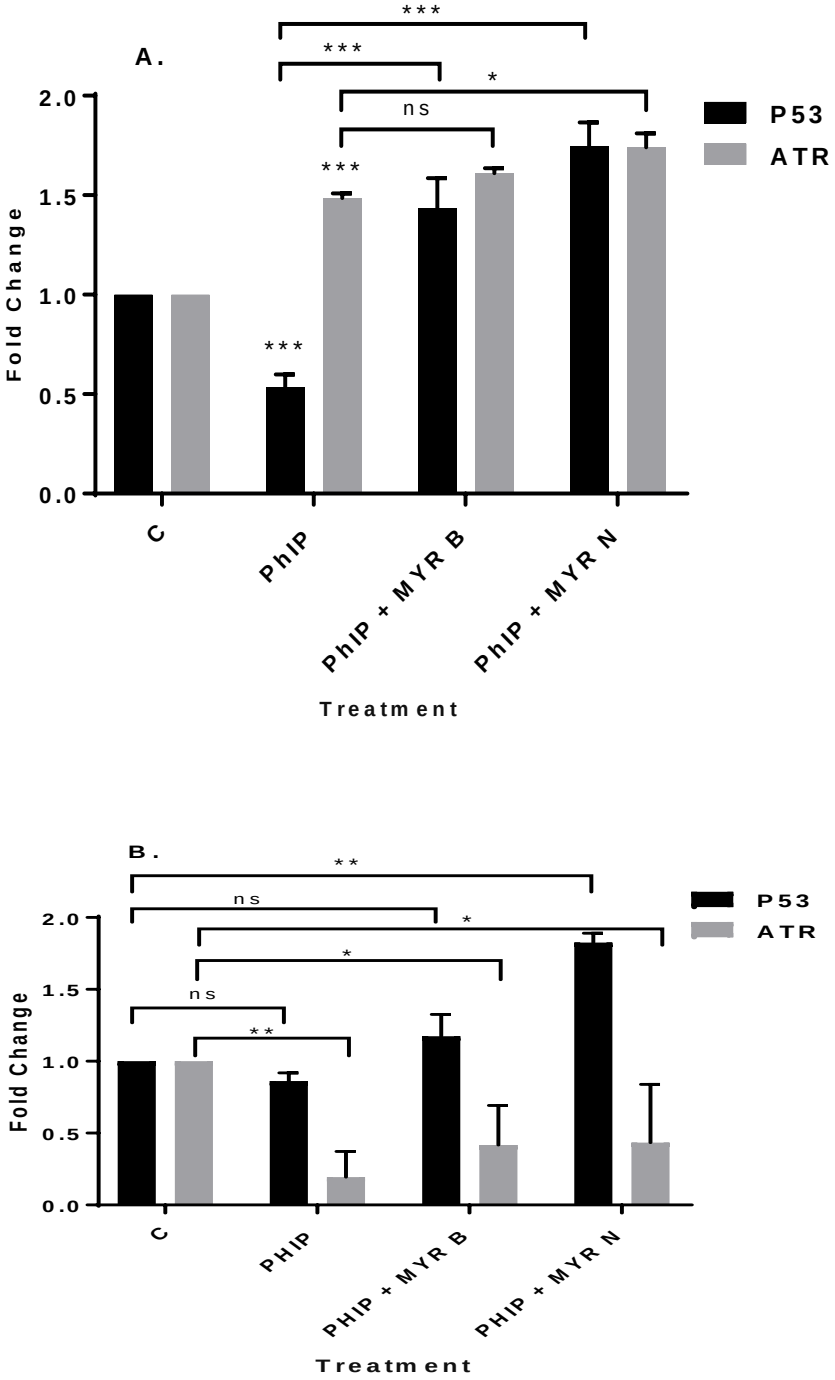
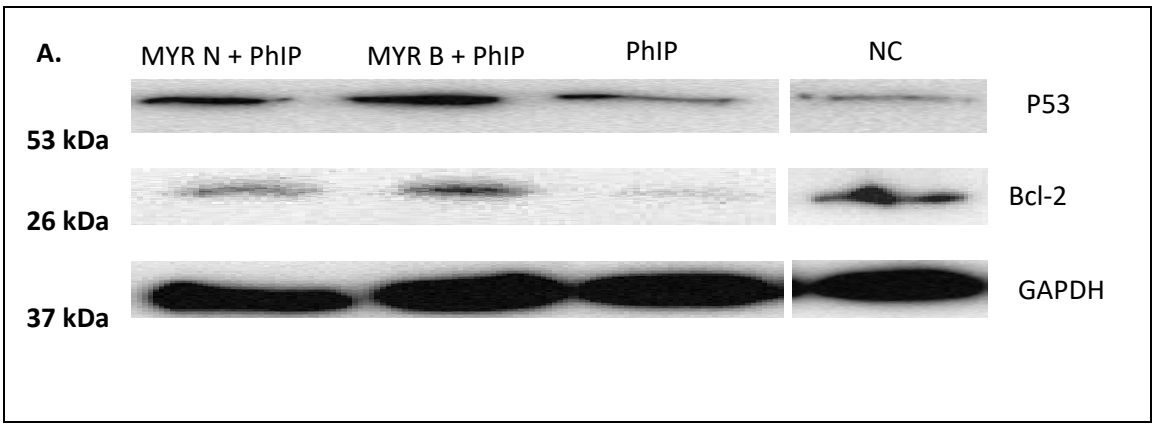


Figure 5.7 The expression of ATR and P53 mRNA in lymphocyte from healthy individuals (A) and pre-cancerous patients (B) after treating with PhIP (100µM), MYR B (10µM) with PhIP and MYR N (20µM) supplemented with PhIP. GAPDH was used as an internal control gene. Gene expression analysis was performed on lymphocytes after 24-hour treatment. Values are the means of three independent experiments, and the error bars represent SDs. (*p<0.01, ** p<0.01, ***p<0.001, ns=not significant). Horizontal lines on the graph represent the difference between the groups. Data were compared against the control (C). All data were normalised against GAPDH reference gene.

5.3.7 Analysis of P53 and Bcl-2 expression in lymphocytes from healthy individuals

As part of this study an investigation was carried out to determine the effect of PhIP treatment and co-supplementation of myricetin bulk and nanoparticles on the protein expression of major anti-apoptotic, Bcl-2 and tumour-suppressor, P53. The expressions were analysed using Western blotting. Results show (Fig 5.8) that P53 and Bcl-2 levels were decreased by 0.6-fold and 0.7-fold, respectively when healthy lymphocytes were exposed to PhIP (100μM). However, combination of PhIP with either MYR B or MYR N significantly increased the expression of both proteins. With MYR B administration, a 1.5-fold increase was observed in P53 and 1.49-fold increase in Bcl-2 expression. MYR N enhanced the levels of former to 1.7-fold and Bcl-2 was up-regulated by 1.52-fold. These results from Western blot analysis indicated that MYR B and MYR N might suppress the mutagenicity caused by PhIP in lymphocytes from healthy individuals by stimulating the expression levels of tumour-suppressor protein, P53 and anti-apoptotic Bcl-2, ultimately causing a protective and anti-tumour effect. Moreover, MYR N has shown more protective results compared to MYR B.



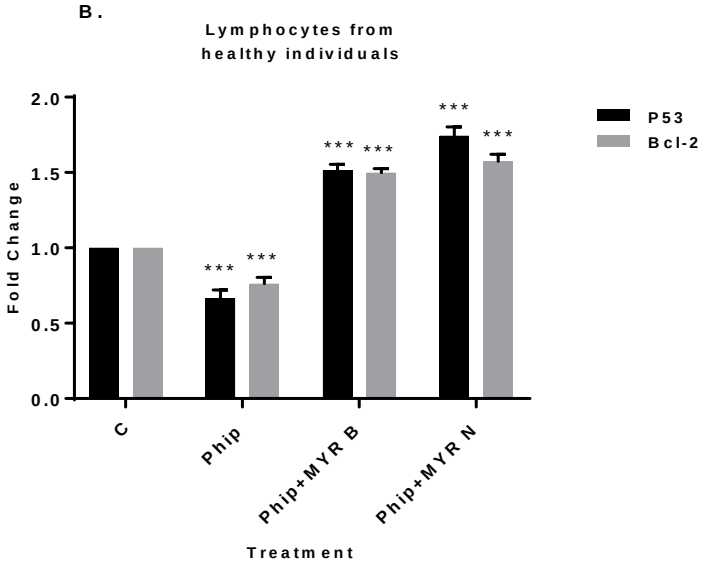


Figure 5.8 Modulating effects of myricetin bulk and nanoparticles on PhIP-triggered alterations in protein expression of P53 and Bcl-2 in healthy lymphocytes (A) Immunoblot analysis of the p53, and bcl-2 proteins in lymphocyte from healthy individuals treated with PhIP (100μM), MYR B (10μM) with PhIP and MYR N (20μM) supplemented with PhIP. P53 and Bcl-2 expression was decreased after PhIP treatment. Supplementation of MYR B and MYR N significantly increased the expression levels of both proteins compared to PhIP alone treated group. GAPDH was used as an internal control protein to normalise the data. **(B)** Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SEM of three experiments. $^{***}P<0.0001$

5.4 Discussion

This study focused on the effects of the food-mutagen, PhIP in lymphocytes from pre-cancerous patients compared to those from healthy individuals and the modulating effects of myricetin against PhIP-induced damage. PhIP has previously been shown to be genotoxic producing DNA adducts (Brown et al., 2001) and contributing towards the formation of dietary cancers (Cheung et al., 2011; Voutsinas et al., 2013). First we demonstrated that PhIP induces significant levels of DNA damage and strand breaks in lymphocytes from healthy and patient groups, determined by the Comet and micronucleus assays ($p<0.001$), supporting previous studies (Mimmler et al., 2016; Buonarati et al., 1990; Boobis et al., 1994). Results from the micronucleus assay have shown a significant induction of MNi formation in BiNC after treatment with PhIP alone (($p<0.001$). A MN formed in BiNC only depicts the damage caused after the treatment, hence reducing the probability of scoring the pre-existing damage (Magdelenova et al., 2012). In micronucleus assay we had to rely on results deducted from 1000 cells scored per treatment group due to manual scoring. However, in future, automated micronucleus scoring techniques could be applied to score a much greater number of cells making the effect more apparent.

Our results confirms that PhIP being a genotoxic agent causes significant DNA damage in lymphocytes from healthy individuals and those from pre-cancerous patients. However, upon treatment with myricetin (MYR B 10μM and MYR N 20μM), PhIP-induced damage was reduced to a substantial level presented by both the Comet and cytokinesis-block micronucleus assay (figure 5.3a, b, 5.4a, b, 5.5). MYR B was effective in reducing the damage moderately ($P<0.004$)

whereas, MYR N was more protective against the damage ($P<0.002$). Both the control lymphocytes and patient lymphocytes showed high sensitivity to PhIP in the Comet assay. Lymphocytes from the pre-cancerous patients had an increased level of basal damage, possibly due to overproduction of ROS and there was a greater difference between the damage measured in lymphocytes treated with PhIP alone and those supplemented with MYR B (10μM) and MYR N (20μM). It is believed that food mutagens damage the DNA by producing ROS and flavonoids act in an anti-oxidant manner to reduce this damage (Kurzawa et al., 2012). Therefore, DNA damage caused by PhIP in lymphocytes from both investigative groups could possibly be because of dual mechanisms: CYP1A2-induced or ROS-induced genotoxicity.

The findings from the current study confirm that myricetin is effectively able to prevent the DNA of lymphocytes from healthy and pre-cancerous patients from PhIP induced-DNA damage. To find whether the protective effects of myricetin are due to its anti-oxidant properties we further investigated the effects of PhIP and co-supplementation of myricetin on the intracellular oxidative defence mechanisms by determining the levels of anti-oxidant enzyme GSH. GSH is major tissue anti-oxidant and the depletion of this crucial enzyme increases the susceptibility to oxidative stress, characterized by the accumulation of ROS (Gawryluk et al., 2011). Glutathione (GSH) is the smallest intracellular protein thiol molecule present in the cells that inhibits ROS (such as free radicals and peroxides) induced cell damage.

GSH is present in two forms, oxidised (GSSG) and reduced (GSH). In healthy cells, the total glutathione pool is mostly in the reduced form (GSH). It is the reduced one which protects against ROS by donating an equivalent ion to the

one being detoxified. However, when cells are exposed to increased levels of oxidative stress, oxidized GSH (GSSG) starts accumulating and the ratio of GSSG to GSH rises. Hence, a bigger ratio of GSSG-to-GSH is an indication of oxidative stress (Roy and Sil, 2012).

Hydrogen peroxide is cleansed through GSH peroxidase (GPx), giving rise to GSSG which is then recycled back to the reduced form by GSH reductase (GR) (Meister, 1988). Therefore, conservation of sufficient GSH levels is crucial for protection against oxidative damage. Our results did not show any effect on GSH levels. However, oxidative DNA damage can be effectively assessed using the FPG Comet assay in future to develop a more precise conclusion on this. FPG Comet can effectively analyze the potential of any compound to induce oxidative damage and DNA strand breaks (Müller, et al., 2013).

Previous studies have shown that the apical DDR kinases such as ATR and ATM can be directly activated by DNA adducts apart from the replication dependent stimulation (Choi et al., 2007; Choi et al., 2009; Kemp et al., 2011). Based on our results from the Comet assay, we established that PhIP induces strand breaks and that myricetin protects against their induction. Hence, to understand the DDR elicited by PhIP and myricetin, we investigated the gene expression levels of ATR kinase and tumour-suppressor gene P53 in lymphocytes from healthy individuals and pre-cancerous patients. Similar patterns of results were obtained for the P53 gene in lymphocytes from both groups. P53 was down-regulated upon PhIP treatment whereas MYR B and MYR N supplementation has shown significant attenuation of PhIP-triggered effects and increased the expression of P53 to substantial levels.

However, PhIP has shown diverse effects on the ATR kinase activity in lymphocytes from healthy individuals compared to those from pre-cancerous patients. It has significantly increased the ATR gene regulation in healthy lymphocytes which was further enhanced by myricetin supplementation ($p<0.001$). This was in agreement with previous studies that upon provoking replication stress, PhIP activates ATR-CHK1 pathway in V79 CS cells (Mimmler et al., 2016).

These results propose that myricetin may protect against the mutagenicity caused by PhIP in healthy lymphocytes by triggering the activation of ATR in P53-mediated DDR pathway. Hence, contribute towards the survival path by the balancing of pro-survival and pro-death signals and through repairing of the cells. To confirm this notion, the post-transcriptional protein levels of P53 and the anti-apoptotic protein Bcl-2 were investigated in healthy lymphocytes. The results demonstrated a significant down-regulation in the expression of both proteins upon PhIP treatment and significant attenuation of the PhIP-induced effects by myricetin supplementation.

On the other hand PhIP significantly ($p<0.01$) down-regulated the ATR kinase activity, in lymphocytes from the patient group. However, myricetin effectively weakened the effects of PhIP and significantly increased ATR regulation. Since the ATR inhibition and increased sensitivity caused by PhIP in patient lymphocytes and the protection shown by myricetin were both dependent on the P53 pathway. This suggests that myricetin could potentially induce apoptosis in PhIP-treated lymphocytes from pre-cancerous patients. Also, the protection depicted by myricetin against PhIP-induced damage may be attributed to its

anti-tumour activity by stimulating the levels of the P53-tumour suppressor gene.

Other possible mechanisms involved are still needed to be investigated further such as the self-repair capacity of lymphocytes by measuring the DNA damage at different time periods using the Comet assay.

Chapter 6: The anti-cancer potential of myricetin in lymphocytes from multiple myeloma cancer patients

6.1 Introduction

Multiple myeloma is a cancer of plasma cells where abnormal plasma cells proliferate and stop producing important antibodies. It is more common in elderly people (Palumbo and Anderson, 2011). Around 1 in 100 people with its precursor disease, MGUS, develop myeloma each year (Rajkumar, et al., 2014). Chemotherapy, steroids, biological therapies and possibly stem cell transplant are the treatment methods currently being utilised for the therapy of multiple myeloma. Initially chemotherapy combined with other treatments works effectively, but myeloma patients usually always have a relapse and these drugs cause various side effects as well including alopecia, nausea, neuropathy. Therefore more effective drugs and novel therapies are required for human cancers (Huang et al., 2015).

Cell cycle is a fundamental process of a cell leading to its division and duplication and ensures the homeostasis in an organ, while a dysregulation in any of its step or components could lead to cancer development. The molecular targets involved in the cell cycle regulatory mechanisms are the main focus of investigational anticancer drugs (Diaz-Moralli et al., 2013). Apoptosis is a mechanism of programmed cell death (PCD) occurring in multicellular organisms. Maintenance of its balance is highly essential for normal growth as excessive apoptosis causes atrophy whereas faulty apoptosis leads to uncontrolled cellular growth which is implicated in various illnesses including cancer. Hence inducing apoptosis may be a promising strategy to overcome various problems related to cancer therapies (Hall et al., 2008). Various processes are involved in inhibition of apoptosis in cancer cells such as P53

mutations and expression of P-glycoprotein. Two major pathways associated with apoptosis are intrinsic (mitochondrial), driven by Bcl-2 family proteins by controlling mitochondrial membrane permeability and release of pro-apoptotic factors and extrinsic (receptor mediated), regulated by tumour necrosis factor-related apoptosis inducing ligand (TRAIL) by mediating its receptors (Brunelle and Letai 2009). Mitochondria play a key role and apoptosis and other cellular metabolic processes. When apoptosis is induced, a variety of metabolic signals produced by mitochondria, cytosol and the membrane are triggered by stimuli. These signals can disrupt the energy metabolisms and modify the expression of Bcl-2 family proteins (Seo, et al., 2003). Upon sensing death signals, mitochondria may release death factors such as cytochrome complex (cyt c) which plays important role in stimulating apoptosis and regulating the oxidative phosphorylation of mitochondria. When released into the cytosol, cyt c binds with Apaf-1 to develop a complex with caspase 9 following the initiation of downstream caspases to slice cellular substrates (Katoh et al., 2004; McDonnell et al., 2003). Reactive oxygen species (ROS), mainly produced by mitochondria intracellularly, are considered to be the second messenger to participate in cellular mechanisms such as apoptosis and proliferation. The anticancer drugs including Taxol induce apoptosis in cancer cell mediated by increasing intracellular ROS levels (Perkins et al., 2000; Varbiro et al., 2001). These data show correlation between ROS, mitochondria and apoptosis but this still needs to be clarified whether cross-talks exist between these components. Anti-apoptotic proteins such as Bcl-2 stop the occurrence of apoptosis by inhibiting the release of cyt c into the cytosol and by maintaining the mitochondrial homeostasis (Gabriel et al., 2003). Pro-apoptotic members of Bcl-2 family proteins such as Bax, forming mitochondrial transmembrane networks, trigger

mitochondrial dependent apoptosis by facilitating the release cyt c and other death factors into cytoplasm (Zhang et al., 2003).

Many research studies associated with flavonoids have discussed and focused on their antioxidant properties but a number of epidemiological studies in various cell lines and models have shown a relationship between flavonoids intake in our diet and low risk of tumorigenesis and their anticancer properties. Flavonoids might have characteristic to prevent inflammation and oxidation, reduce cell proliferation and angiogenesis and stimulate apoptosis (Gates et al., 2009). Past studies have shown, *in vitro* cytotoxicity exhibited by flavonoids in various cell lines, including colon cancer cells (Takagaki et al., 2005), prostate cancinoma cell (Shukla and Gupta 2006), hepatoma cells (Chiang et al., 2006) and cervical carcinoma cells (Zheng et al., 2005).

Many past studies have demonstrated that myricetin induces apoptosis in various cancer cell lines comprising hepatoma, colon carcinoma cells, oesophageal and pancreatic cancer (Phillips et al., 2011; Zhang et al., 2013; Zang et al., 2014). It has been previously shown that flavonoids have the ability to reduce the viability of tumour cells by the induction of apoptosis and that both ROS dependent and independent pathways may be involved (Chen et al., 2003; Ko et al., 2004). In the literature, there is no evidence of research on the mechanism of action of myricetin bulk and nanoparticles in lymphocytes of multiple myeloma patients. We have chosen MM as target blood cancer due to its association with MGUS. MGUS is a common condition where a small number of plasma cells start producing paraprotein, and it has been shown that patients with MGUS have a greater risk of developing MM (Rajkumar et al., 2014) and our initial work involved those pre-cancerous patients who have this

condition. As MM originates from the plasma cells which are a type of B lymphocytes, hence, in our current study we used surrogate cells, lymphocytes, as model cells to examine the effects of different particle sizes of myricetin i.e. the bulk (MYR B 10μM) and nanoparticle form (MYR N 20μM), from multiple myeloma patients and also investigate the molecular mechanisms involved in their effects. We further investigate the effects of myricetin on intracellular ROS levels in lymphocytes.

6.2 Materials and Methods

6.2.1 Cell culture and reagent

Multiple myeloma patient blood was kindly provided by the Department of Haematology, Bradford Royal Infirmary BRI, Bradford, UK. Normal healthy blood was donated by healthy individuals. Ages for all individuals from both investigative groups were best matched and the Comet assay results show no significant difference in DNA damage due to age factor (figure 2.1, chapter 2). Lymphocytes from all the blood samples (table 6.1 and 6.2) were isolated and maintained in RPMI-1640 medium (Sigma Aldrich, UK), Supplemented with 10% foetal bovine serum FBS (Invitrogen, UK) and 1% penicillin streptomycin (Invitrogen, UK) in a humidified incubator at 5% CO_2 and at 37^0C. Myricetin was purchased from Sigma Aldrich, UK and was dissolved in excipient mixture to produce its bulk and nano forms. The primary antibodies against P53, Bcl-2, Bax and GAPDH were purchased from Abcam, Cambridge, UK.

No	Age	Ethnicity	Gender	Smoking history	Family history
1	60	ASIAN	M	YES	NONE
2	59	CAUCASIAN	F	YES	NONE
3	61	CAUCASIAN	F	NO	NONE
4	52	CAUCASAIN	F	NO	NONE
5	60	CAUCASIAN	F	NO	NONE
6	55	CAUCASIAN	M	NO	NONE

Table 6.1 Brief information of blood samples from healthy individuals used in this chapter

No	Age	Ethnicity	Gender	Smoking history	Family history	Medical condition
1	55	CAUCASIAN	F	NO	NONE	MULTIPLE MYELOMA
2	56	CAUCASIAN	M	NO	PANCREATIC CACNER	MULTIPLE MYELOMA
3	79	CAUCASIAN	M	NO	BREAST CANCER	MULTIPLE MYELOMA
4	77	CAUCASIAN	F	NO	OVARIAN AND BREAST CANCER	MULTIPLE MYELOMA
5	70	CAUCASIAN	M	NO	NONE	MULTIPLE MYELOMA
6	87	CAUCASIAN	M	NO	NONE	MULTIPLE MYELOMA

Table 6.2 Brief information of blood samples from MM patients used in this chapter

6.2.2 Procedures used

For detailed procedures of cell viability using MTT, determination of ROS production, DNA damage assessment using the Comet assay, Western blot analysis, and the real-time RT-PCR analysis, please refer to chapter 2. The primers used for RT-PCR are listed in table 6.3.

6.2.3 Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). Graph Pad prism was used to perform statistical calculation. The results were analysed

using t-tests and one-way analysis of variance (ANOVA) to test differences between each treatment and control. A p-value of <0.05 was considered statistically significant.

Genes	**Primer Sequence 5'-3'**
Bcl-2	CTTTGAGTTCGGTGGGGTCA GGGCCGTACAGTTCCACAAA
Bax	AAGCTGAGCGAGTGTCTCAAG CAAAGTAGAAAAGGGCGACAAC
P53	CTCCTCAGCATCTTATCCGAGT GCTGTTCCGTCCCAGTAGATTA
GAPDH	TGCACCACCAACTGCTTAG AGTAGAGGCAGGGATGATGTTC

Table 6.3 Primers used for Real-Time PCR analysis

6.3 Results

6.3.1 MTT assay

The MTT assay was performed to assess the cytotoxic effects of both forms of myricetin and the results demonstrated a time and dose-dependent cytotoxicity in human lymphocytes. The effects of MYR B (10-40μM) and MYR N (10-40μM) on the viability of healthy lymphocytes at different times (1, 24 and 48hrs) compared to the respective untreated group have already been shown in figure 3.4 A where MYR B and MYR N exhibited a time and concentration dependent decrease in cell viability. MYR B (40μM) and MYR N (40μM) reduced the viability in lymphocytes from healthy individuals to 88% and 86% respectively, after a 48 hours treatment. Neither of the concentrations of MYR B and MYR N reduced viability less than 80% in healthy group. However, MYR B and MYR N at 40μM reduced the viability to 68% and 51% respectively after 48 hours of

treatment (Fig 6.1), in lymphocytes from multiple myeloma patients. Lower concentrations (10 and 20μM) of both forms of myricetin did not reduce the viability less than 80% in lymphocytes from the patient group. The effects of the excipient mixture (the vehicle mixture for chemical preparation) were also considered to exclude any errors. Myricetin in both forms showed higher level of cytotoxicity in lymphocytes from myeloma cancer patients than in those from healthy individual at higher concentrations indicating that cancer cells are more sensitive to myricetin bulk and nanoparticles than the healthy ones possibly due to the compromised defense and repair mechanisms owing to the disease state. Hence, the current study was conducted using non-toxic concentrations of MYR B and MYR N so that any of the results obtained are not due to artefact of toxicity. The non-genotoxic concentrations used for MYR B and MYR N are 10μM and 20μM respectively, determined by dose response curve (Figure 3.3) throughout the current *in vitro* study.

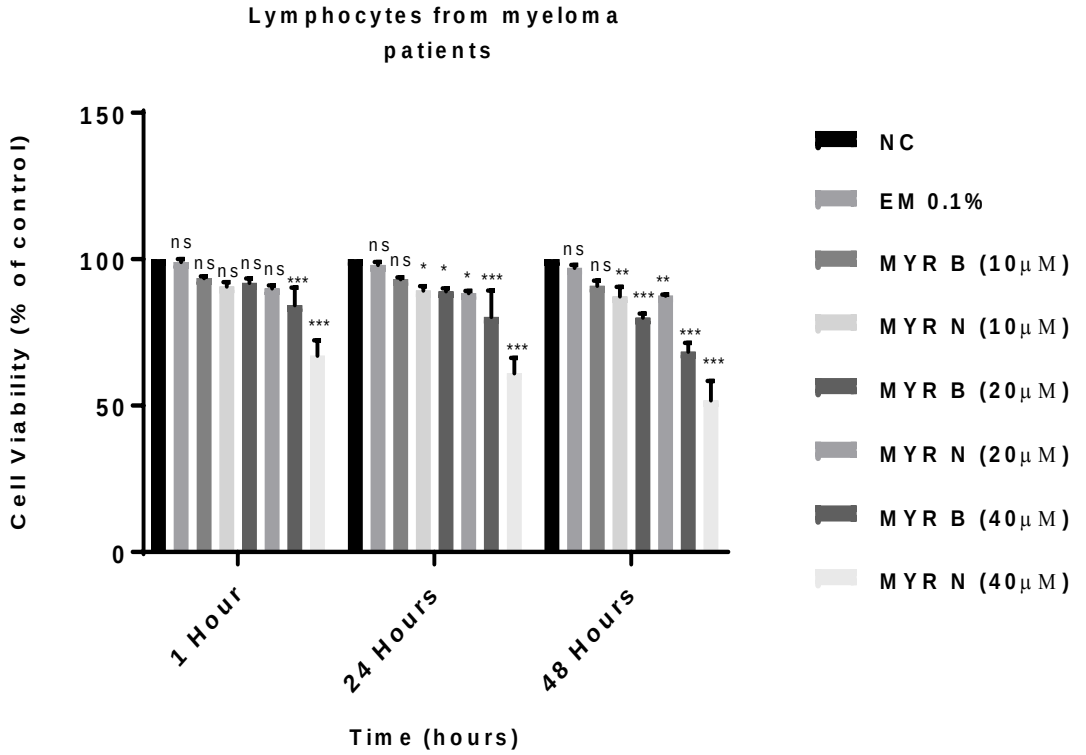


Figure 6.1 Determination of cell cytotoxicity by MTT based assay. Lymphocytes from MM patients showing cell viability after treatment with different concentrations of myricetin bulk and nanoparticles Cell cytotoxicity was expressed as % of the control for 1, 24 and 48h. The treatment groups included untreated (NC), MYR B (10μM, 20μM and 40μM), MYR N (10μM, 20μM and 40μM) and EX (excipient mixture 0.1%). The best concentrations for MYR B and MYR N were determined by dose response curves. Values are the means of three independent experiments and the error bars represent SDs. (ns=not significant, *P<0.01, **P<0.003, ***P<0.0001)

6.3.2 The Comet assay

DNA damage and strand breaks formation caused by MYR B (10μM) and MYR N (20μM) in lymphocytes from healthy individuals and myeloma cancer patients was assessed at basal levels without supplementation of any stress inducer using the Comet assay. Fig 6.2 (A, B) shows no significant DNA damage induced by MYR B and MYR N in healthy lymphocytes. However, results shown in fig 6.3 (A,B) indicate that DNA damage was induced in the patient group by both forms of myricetin as compared to the untreated group but the levels were not significant when presented using two parameters; % Tail DNA and Olive Tail moment.

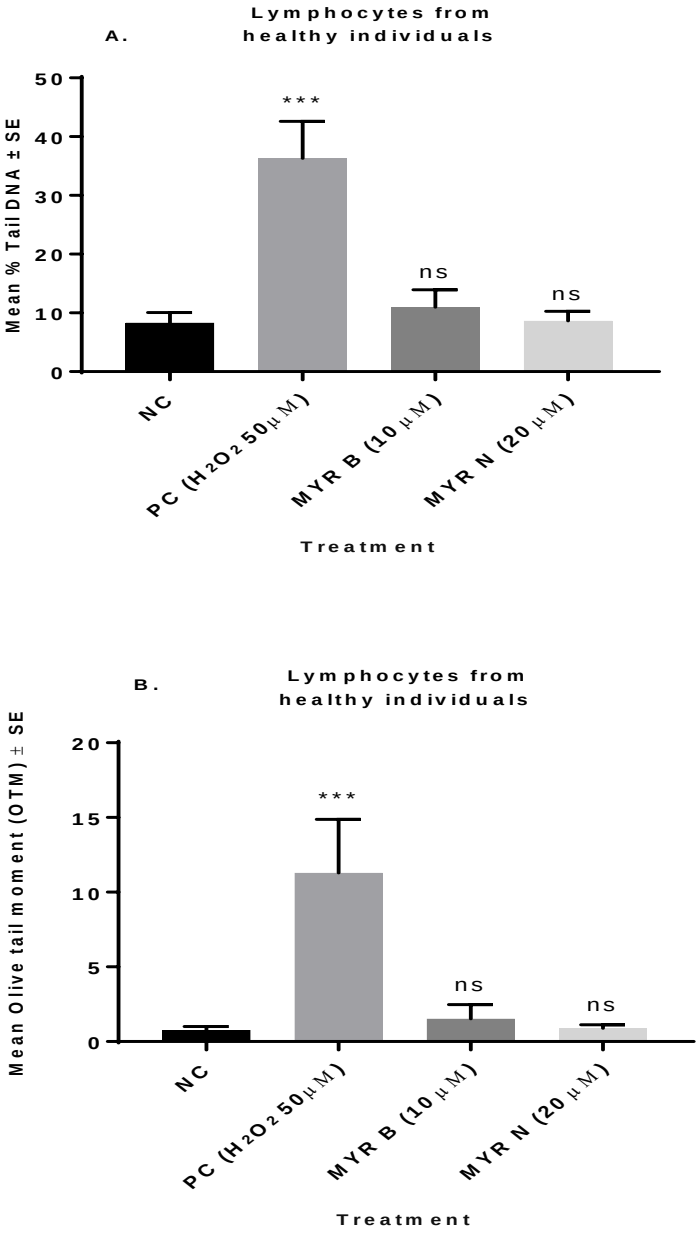
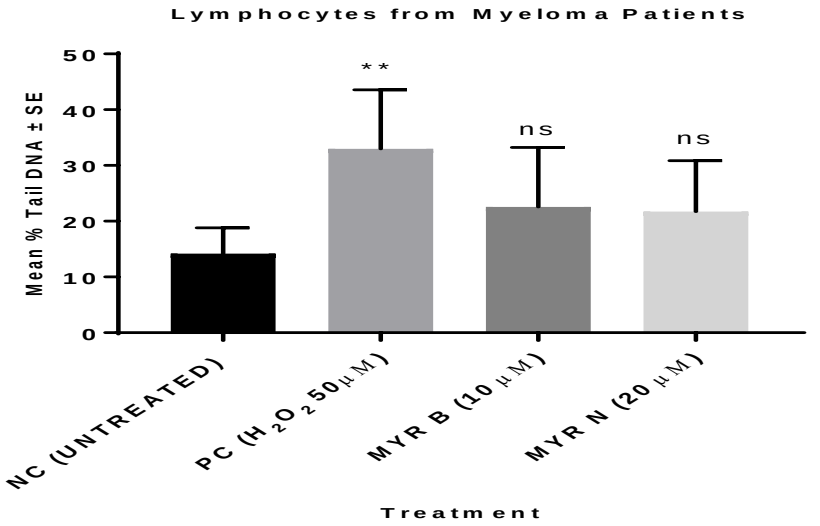


Figure 6.2 The response of bulk and nano forms of myricetin on lymphocytes DNA from healthy individuals using %Tail DNA (A) and olive tail moment (OTM) (B) The figure shows the mean of experiments in 6 individuals, counting 100 cells each for four different groups of treatments; an untreated lymphocyte group (NC), positive control (PC) 50µM H_2O_2, myricetin bulk (MYR B 10µM) and myricetin nano (MYR N 20µM). All treatment groups were compared to the NC group. The mean control value was 9 and the PC had the maximum mean value of 36 for % Tail DNA.($^{***}P<0.001$,ns means not significant analysed by one way ANOVA.. The mean control value was 0.7 and the PC had the maximum mean value of 11 for OTM.



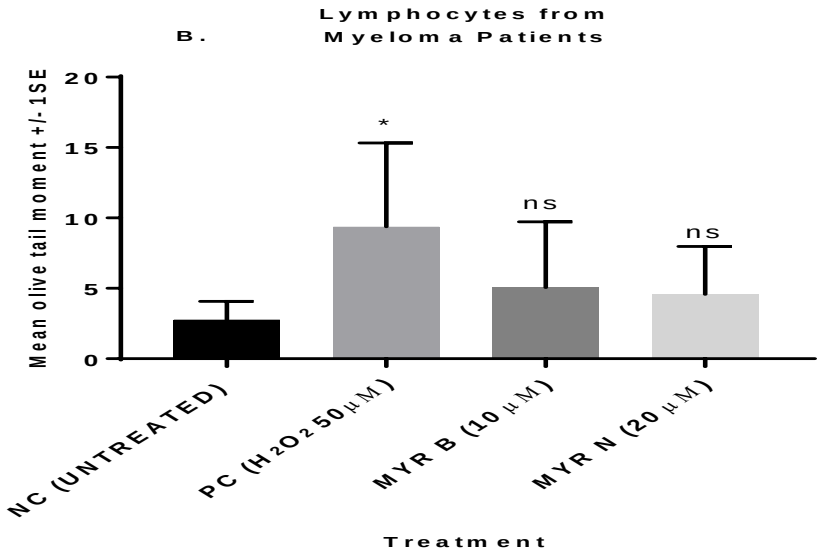


Figure 6.3 The response of bulk and nano forms of myricetin on lymphocytes DNA from myeloma patients using %Tail DNA (A) and Olive tail moment (OTM) (B) The figure shows the mean of experiments in 6 individuals, counting 100 cells each for four different groups of treatments; an untreated lymphocyte group (NC), positive control (PC) 50µM H_2O_2, myricetin bulk (MYR B 10µM) and myricetin nano (MYR N 20µM). All treatment groups were compared to the NC group. The mean control value was 14 and the PC had the maximum mean value of 32 for % Tail DNA. The mean control value was 2.7 and the PC had the maximum mean value of 9.3 for OTM. $^{**}P<0.0001$,$^{*}P<0.001$, ns means not significant) analysed by one way ANOVA.

6.3.3 Real-time PCR

Based on the previous results from MTT and the Comet assay, we piloted that myricetin bulk and nanoparticle forms do not induce statistically significant DNA damage in lymphocytes from myeloma cancer patients compared to those from healthy individuals. To study the molecular responses mediated by myricetin on the apoptotic pathway, we investigated the effects of myricetin on gene expression of P53, Bax and Bcl-2, in lymphocytes from healthy individuals and myeloma cancer patients at the mRNA level. Real-Time PCR analysis was conducted for the apoptosis related genes. The results from the real-Time PCR analysis show that MYR B (10μM) and MYR N (20μM) regulate the expression of some genes involved in intrinsic apoptosis at mRNA level. The expression of Bcl-2/Bax ratio increased in healthy lymphocytes. Bax was significantly down-regulated by 0.6-fold and 0.74-fold by MYR B and MYR N, respectively while Bcl-2 was up-regulated by 1.2-fold with MYR B and minutely decreased by 0.8-fold with MYR N. However, P53 gene was insignificantly down-regulated (Fig 6.4). PCR analysis shows a different results pattern in lymphocytes from myeloma cancer patients. The expression of the tumour suppressor genes, P53 and pro-apoptotic gene, Bax, was significantly up-regulated by 2.0-fold and 1.9-fold respectively, upon treatment with MYR N. When treated with MYR B, the former was up-regulated by 1.4-fold and the latter by 1.7-fold. The expression of anti-apoptotic gene, Bcl-2, was significantly down-regulated by 0.75-fold with MYR N and by 0.7-fold with MYR B (Fig 6.5). Results show the alterations in these three genes (P53, Bax, Bcl-2), indicating the apoptotic potential of myricetin in lymphocytes from myeloma cancer patients might be dependent on expression levels of P53 gene.

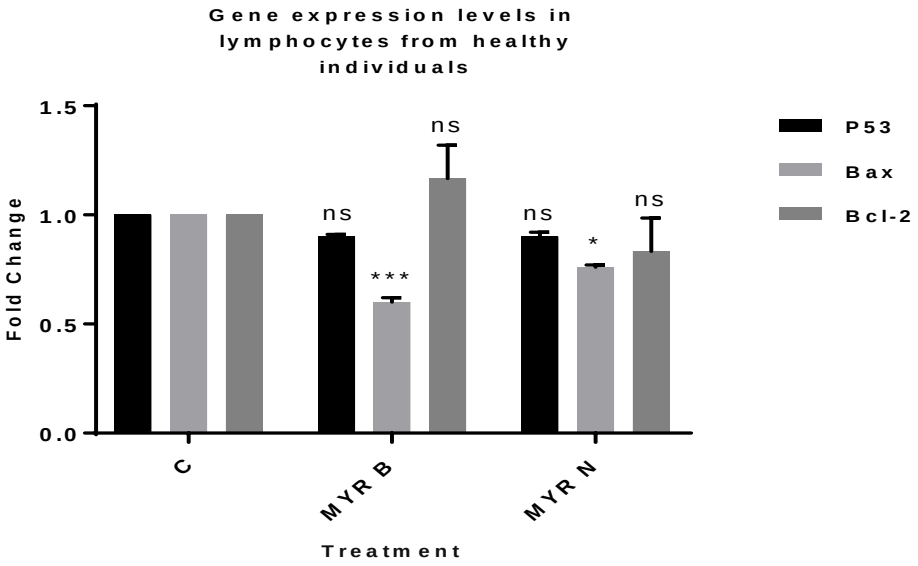


Figure 6.4 The effect of MYR B and MYR N on the gene expression levels of Bax and Bcl-2 and P53 in lymphocytes from healthy individuals. All results were compared against control group (C) and normalised against the internal gene, GAPDH, Bax is significantly decreased by both MYR B and MYR N treatments. However these treatments did not show any effect on P53 and Bcl-2 expression. Values are the means of three independent experiments. (ns=not significant, *P<0.01, ***P<0.002)

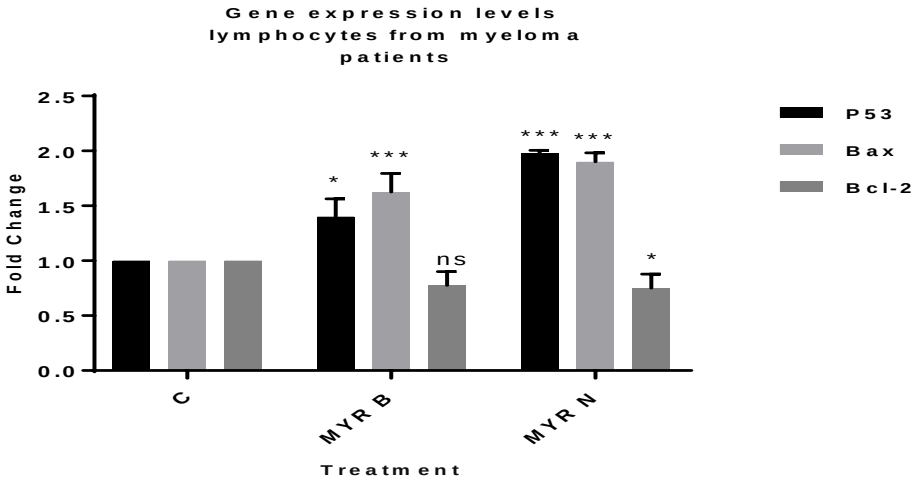


Figure 6.5 The effect of MYR B and MYR N on the gene expression levels of Bax and Bcl-2 and P53 in lymphocytes from untreated multiple myeloma (MM) patients. All results were compared against control group (C) and normalised against the internal gene, GAPDH where n=4. The m RNA P53 and Bax expression increased with both form of myricetin whereas Bcl-2 expression decreased. (ns=not significant, *P<0.01, ***P<0.001)

6.3.4 Effects of myricetin bulk and nanoparticles on the proteins involved in intrinsic apoptotic pathway in isolated lymphocytes

A series of Western blot experiments were piloted to find whether the changes in the gene expression of Bcl-2, Bax and P53 at mRNA level ultimately leads to the alterations at the protein expression level.

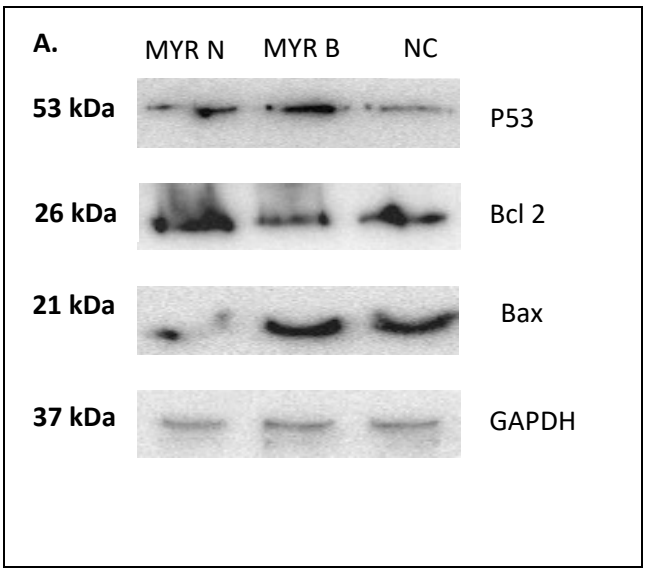
6.3.4.1 Western blotting

An investigation was carried out in order to find whether the mitochondrial dependent intrinsic pathway was involved in apoptosis induction potential of myriceitn. The protein expression of major pro-apoptotic and anti-apoptotic protein levels, Bax and Bcl-2 respectively were analysed using Western blotting. Results show (Fig 6.6 A, B) that Bcl-2 levels were increased by 3.4-fold with MYR B and 2.1-fold with MYR N, in lymphocytes from healthy individuals. However, Bax seemed to be down-regulated, by 0.6-fold after exposing to MYR B and 0.4-fold with MYR N treatment. Fig 6.7 (A, B) show Bax levels were significantly increased by 2.9-fold with MYR B and 3-fold with MYR N, in patient lymphocytes. However, Bcl-2 seemed to be significantly down-regulated, by 0.6-fold after exposing to MYR B and 0.9-fold with MYR N treatment which is not significant. These results from Western blot analysis indicate that MYR B (10μM) and MYR N (20μM) might have a potential to induce apoptosis in lymphocytes from myeloma patients by altering the ratio of Bcl-2 family proteins expression.

6.3.4.2 Role of P53 in myricetin-mediated regulation of apoptosis-related proteins

P53 is a tumour-suppressor protein which plays a vital role in several cellular processes including apoptosis and angiogenesis (Darcy, et al., 2008). Therefore

we examined P53 protein to verify if myricetin bulk and nanoparticles mediate effects in lymphocytes of MM patients through this protein. We found (Fig 6.6 A,B) that the P53 protein was up-regulated in the lymphocytes of healthy individuals after treatment with MYR B and MYR N, 2.2-fold and 1.8 fold, respectively when compared to the control. A representative immunoblot is shown in figure 6.6A, whilst the graph in figure 6.6B is average of three experiments. Fig 6.7 A, B. shows that p53 was up-regulated in the lymphocytes from MM patients as well, after treatment with MYR B and MYR N, 2.2-fold and 3.2-fold, respectively when compared to the control. Increased *in-vitro* expression of P53 in lymphocytes, when exposed to myricetin, indicates that myricetin exhibits anti-cancer potential in lymphocytes from MM patients. All the results from Western blot analysis for these proteins were in agreement with the Real-time PCR data for the related genes.



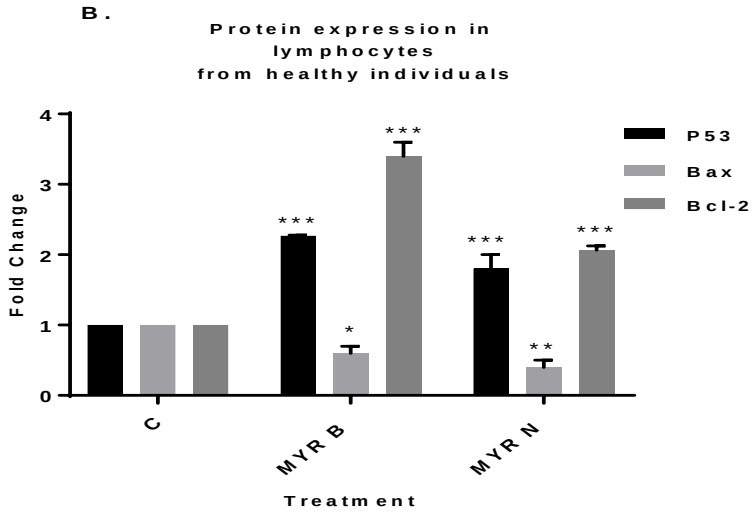
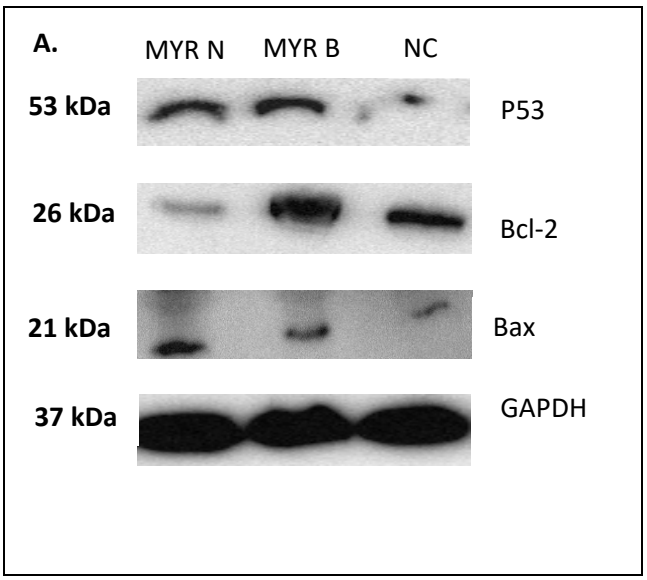


Figure 6.6 The effect of Myricetin Bulk and nanoparticles on apoptosis-related proteins in healthy lymphocytes (A) Immunoblot analysis of the P53, Bax and Bcl-2 proteins in lymphocyte from healthy individuals treated with MYR B and MYR N. P53 and Bcl-2 expression was increased while Bax expression decreased. GAPDH was used as an internal control protein to normalise the data. **(B)** Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SD of three experiments. (*P<0.01, **P<0.002, ***P<0.0001)



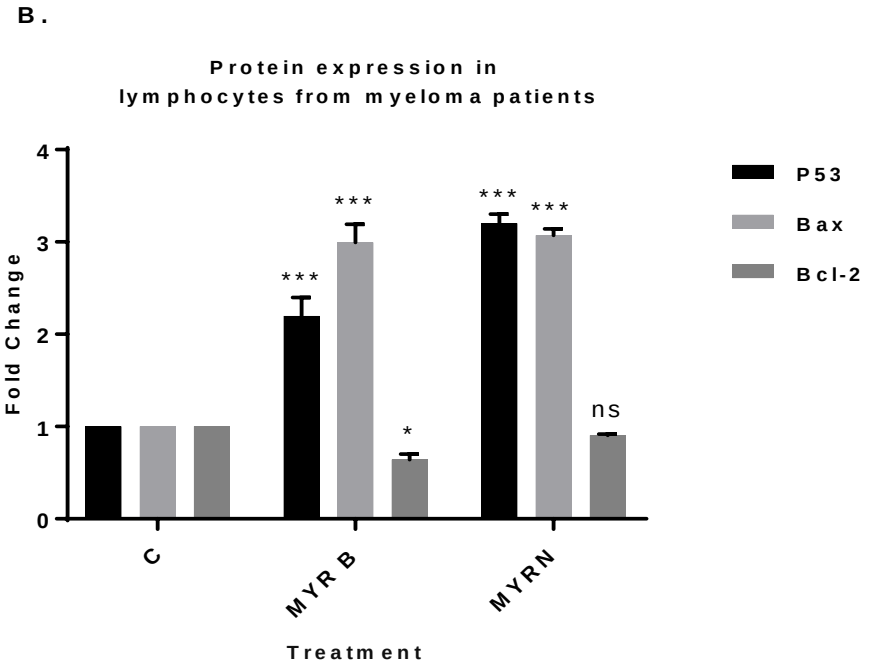


Figure 6.7 The effect of Myricetin bulk and nanoparticles on apoptosis-related proteins in lymphocytes from myeloma patients (A) Immunoblot analysis of the P53, Bax and Bcl-2 proteins in lymphocyte from MM cancer patients treated with MYR B and MYR N. P53 and Bax expression was increased whereas Bcl-2 expression was decreased. GAPDH was used as an internal control protein to normalise the data. **(B)** Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SD of three experiments. (*P<0.01, ***P<0.0001, ns=not significant)

6.3.5 Myricetin and ROS production

We investigated if ROS has a role in alteration of apoptotic genes in lymphocytes from MM cancer patients *in vitro* compared to those from healthy individuals. Results demonstrate that MYR N (Fig 6.8) has significantly increased the endogenous peroxide levels indicated by an increase in fluorescence intensity compared to the untreated group. However, results indicate that ROS levels are decreased upon MYR B and MYR N treatment to statistically non-significant levels in lymphocytes from healthy individuals. These data suggest that the alteration in the ratio of Bcl2/Bax by myricetin in lymphocytes from MM patients might be dependent on ROS production. Data also suggests pro-oxidant activity of myricetin in lymphocytes from MM cancer patients.

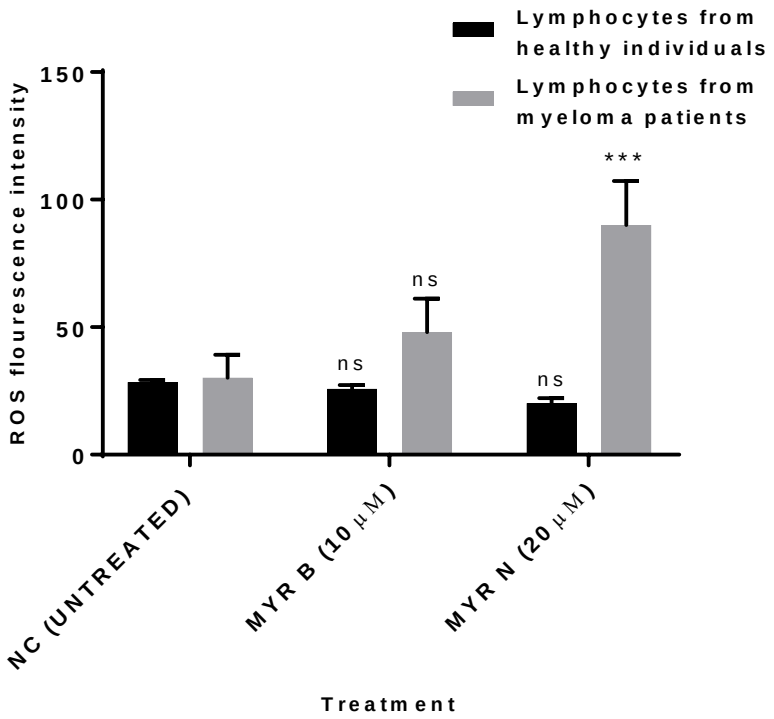


Figure 6.8 Myricetin induce Reactive oxygen species (ROS) in lymphocytes from MM patients. Isolated lymphocytes from healthy individuals and MM cancer patients were treated with MYR B (10μM) and MYR N (20μM) while compared against the respective untreated group (0 μM myricetin). The intracellular ROS levels were assessed using DCF-DA assay. Values are the means of three independent experiments and the error bars represent SDs, analysed by the two-way ANOVA. (ns=not significant, $^{***}p<0.001$) $p<0.05$ values were considered significant

6.4 Discussion

Cancer is a life-threatening disease in current world and its occurrence is alarmingly increasing with passage of time and the main therapeutic strategies being utilised are chemotherapy and radiotherapy. Due to high rates of adverse effects and chemo resistance related to chemotherapy, there is an urgent need to develop some novel drugs which could address these issues (Yang et al., 2011). Various flavonoids have been shown to induce cytotoxicity in different cancer cell lines such as cervical (Chiang et al., 2006), lung (Leung et al., 2007), colon (Chen et al., 2004) and prostate cancer cells (Vijayababu et al., 2006). However, the potential protective effects of myricetin on the lymphocytes from MM cancer are rarely reported. Similarly many past studies have reported myricetin causing apoptosis in various cancers including colon, hepatoma, esophageal and pancreatic etc. (Zhang et al., 2013; Phillips et al., 2011; Zang et al., 2014; Kim et al., 2014). Conversely, its effects on MM are currently not known.

Myricetin is found extensively in vegetables and fruits and has health beneficial effects but its apoptotic effects are still unclear (Pérez-Cano and Castell 2016). It was found that myricetin in both forms (MYR B 10μM, MYR N 20μM) has not induced any statistically significant DNA damage in lymphocytes from MM patients compared to the healthy lymphocytes determined by the Comet assay results. The results from the Comet assay also revealed that there were no significant differences in DNA damage of patient group due to the age factor (figure 2.1).

Apoptosis, a process of programmed cell death is an important factor to get rid of cancer cells. A mechanism through which cancer cells form resistance to

chemotherapy is by avoiding apoptosis and enhancing proliferation. Two main pathways involved in apoptosis are; intrinsic (mitochondrial dependent) and extrinsic (receptor-mediated) pathways. Upon activation of the intrinsic pathway, the mitochondria permeability and the release of cytochrome complex (cyt c) into the cytosol increase. This forms a multi-protein complex called apoptosome which then triggers the initiation of caspase-9. The Bcl-2 family proteins play a vital role in regulation of this process by monitoring the permeability of the mitochondrial membrane and through the release of pro-apoptotic elements. The fate of cells to undergo apoptosis or not mainly depends on the balance between the pro (e.g. Bax, Bad) and anti-apoptotic (e.g. Bcl-2, Bcl-xl) factors of the Bcl-2 family (Brunelle and Letai, 2009). Extrinsic apoptotic pathway involves receptor DR5, caspases 8 and 10 regulation. Whether the apoptosis is initiated by intrinsic or extrinsic pathway, it ultimately leads to activation of caspase 3 and 7 respectively, concluding the apoptosis. In the current study myricetin bulk and nano forms were found to significantly up-regulate Bax/Bcl-2 protein ratio in lymphocytes from MM patients (likely to affect apoptosis) but not in those from healthy individuals. This might be due to differential effects of myricetin on healthy and patient lymphocytes. This suggests that myricetin could potentially induce intrinsic mitochondrial dependent apoptosis in lymphocytes from MM patients. In future, the possibility of looking at apoptotic cell numbers using various techniques including TUNEL assay and flow cytometry analysis of apoptotic markers following treatment of lymphocytes with myricetin, could help to confirm the apoptosis-inducing potential of myricetin bulk and nano forms in lymphocytes from MM patients.

Our results are consistent with a previous study where myricetin induced apoptosis in colon cancer cells by increasing the ratio between Bax and Bcl-2 protein levels (Kim et al., 2014).

The functional association between the ROS and mitochondria are not understood but various studies have shown the positives and negatives on this aspect. A variety of stimuli triggers cyt c release and apoptosis through ROS production. However, ROS also play a mitogenic role by inducing proliferation and protecting cells against oxidative stress-induced apoptosis (Kim et al., 2001; Kops et al., 2002). Hence, data suggest a dual function of ROS. In our study the increase in the ratio between Bax and Bcl-2 in lymphocytes from myeloma patients was also dependent on ROS production as ROS levels increased slightly with myricetin bulk and significantly increased with myricetin nano treatment when compared to the untreated control and healthy control group (fig 6.8).

P53, a multiple functional tumour suppressor protein plays a key role in regulation of various cellular processes including apoptosis, controlling cell cycle progression, transcription, angiogenesis, DNA repair and senescence (Darcy et al., 2008). In presence of DNA damage, P53 inhibits the proliferation of damaged cells by different regulatory processes (Haupt et al., 2003; Haupt and haupt, 2017). In a number of human cancers, this house-keeper gene is inactivated (loss of normal function) by mutation leading to unmonitored cell growth. Genetic lesions of P53 occurring one on each of the two alleles in every cell, leads to complete inactivation of P53 and development of a tumour. Hence the cancer cell is usually a condensed hemizygous mutant for P53 gene. The alterations in the P53 gene are connected with failure in chemotherapy and radiotherapy in various human cancers (Kong et al., 2012). Reintroduction of

wild type P53 into cancer cells which possess its mutant form, leads to either apoptosis or cell cycle arrest (Martinez et al., 1991). The ability of P53 to inhibit cell proliferation, causing cell cycle arrest and apoptosis depends mainly on its binding to the target genes involved in these processes (Moll and Slade 2004). Recently, the concept of cell-cycle regulated apoptosis is gaining increasing emphasis for eliminating cancer cells (Calcabrini et al., 2006). Increasing the amount of P53 may be a new strategy for treating cancer as it regulates the intrinsic apoptosis pathway such as Bax (kuo et al., 2006). Flavonoids such as myricetin are considered as promising chemicals with ability to selectively eliminate cancer cells by cell-cycle arrest or/and apoptosis (Choi et al., 2001; Wang et al., 2004; Hsu et al., 2004; Ujiki et al., 2006; Mu et al., 2007; Shukla and Gupta, 2007; Pérez-Cano and Castell 2016).

In the present study, myricetin bulk and nanoparticles were observed to increase the level of P53 gene in lymphocytes from myeloma patients at mRNA and protein level (fig 6.5, 6.7).

Also, the structural properties of myricetin (OH groups at C3', 4' and 5') might have possibly played a role in its anticancer and apoptosis-inducing potential, supported by a past study (Ko et al., 2004).

In conclusion, myricetin bulk and nanoparticles have exhibited anticancer potential and pro-oxidant activities in lymphocytes from MM cancer patients. It has altered the levels of Bcl-2 family proteins, regulated by P53 and somehow dependent on ROS production. This is indicative of the potential of myricetin to induce apoptosis in lymphocytes from MM patients and to be possibly used as an anticancer drug for MM. Moreover, MYR N (20μM) has collectively shown more effective responses than its larger particles (i.e. MYR B 10μM) in lymphocytes from both investigated groups.

Chapter 7: General discussion and future work

7.1 General Discussion

The present study was conducted to investigate the genoprotective and genotoxic effects of myricetin bulk and nano forms in lymphocytes from pre-cancerous and MM patients while compared to those from healthy individuals. Also, to determine the anti-oxidant potential of myricetin bulk and nano forms in lymphocytes form pre-cancerous patients and healthy individuals by measuring their effect against ROS-induced oxidative stress. The modulating effects of myricetin against PhIP-induced DNA damage were also studied.

Cancer presents a major public health threat in both the developed and developing world. Prevention of cancer, by use of natural dietary constituents, is a promising strategy to overcome the dogma linked with a constantly growing number of cancers worldwide (Tsao and Edward, 2004). Plant derived phenolic compounds are probably the most studied natural compounds due to their vast potential health beneficial effects, as proposed by various studies (Del Rio et al., 2010). Flavonoids, tanins and phenolic acids are major classes of phenolics and reflect the main ones consumed in our diet (Balasundram et al., 2006). Flavonoids constituting the largest group of plant phenolics exhibit a diverse spectrum of pharmacological activities including their possible anti-cancer and anti-oxidant property (Bhuyan and Basu, 2017). A positive association has been shown in the past between the flavonoids enriched diet and lower risk of many cancers including colon, breast and prostate cancer (Batra and Sharma, 2013). Flavonoids are subdivided into different classes; flavones, flavanols, flavonols, anthocyanins, flavonones and isoflavonols depending on their chemical structure. Among these, flavonols, anthocyanins and flavanols are those occurring most in the human diet (obtained from plant origins) and could be responsible for prevention against cancer development, neurodegeneration and

cardiovascular diseases (Bazzano et al., 2002; Xiao et al., 2011; Atmani et al., 2009; Fang et al., 2010).

Myricetin (3,3',4',5,5',7-hexahydroxyflavone), a major flavonol, mainly found in red wine, berries and vegetables, has been recently utilised as a health supplement owing to its significant anti-oxidant and anti-tumour activities (Miean and Mohamed, 2001; Hobbs et al., 2015; Miyazaki et al., 2017). Myricetin has been extensively studied investigating its anti-cancer potential and its mechanism of action against fighting different cancers (Maggiolini et al., 2005; Devi et al., 2015; Lu et al., 2006; Kumamoto et al., 2009; Sun et al., 2012,). For example, myricetin induces cell death in colon cancer cells through the Bax/Bcl-2 dependent pathway (Kim et al., 2014). It prevents MMP- 2 protein expression and enzyme activity in colorectal carcinomas cells (Ko et al., 2005). Moreover, myricetin suppresses and attenuates the neoplasm transformation of the tumour cells by interacting with various oncoproteins such as protein kinase B (PKB) (Kumamoto et al., 2009; Sun et al., 2012; Devi et al., 2015). Studies have shown myricetin to display an anti-HIV-1 activity on HIV-1 infection (Pasetto et al., 2015) and therapeutic potential against diabetes mellitus (Li and Ding, 2012). The diverse activities and effects exhibited by myricetin indicate that it interacts with numerous target proteins in divergent cell types.

The effects of the major dietary factors (fruits, vegetables, meat, and fat) and other nutrients (such as vitamins, phytochemicals, minerals etc.) on carcinogenesis is a complex area. As well as, studying the association between these dietary essentials and the risk of cancer development, it is important to determine the underlying processes through which these elements affect carcinogenesis. From this perspective the Comet assay presents an excellent method and is a relatively easy, simple, sensitive and inexpensive way to

determine DNA damage and repair. Moreover, it can be used to study the effects of nutrition and diet on cancer. The Comet assay with its various modifications can be potentially used to study the nutritional-related biomarkers of cancer and can possibly detect cancer at an early stage in every individual (Wasson et al., 2008; Anderson et al., 2018). Therefore, we used this highly sensitive and reliable method to study the basal effects of myricetin (bulk and nanoparticles form) on lymphocytes from pre-cancerous (chapter 3) and multiple myeloma patients (chapter 6) compared to those from healthy individuals. Also, to determine the repair capacity of lymphocytes and modulating effects of myricetin on H_2O_2 (chapter 4) and PhIP-induced DNA damage (chapter 5).

Since many studies have proposed that the combination of the Comet assay along with the CBMN assay is best test to evaluate the mutagenic potential and possible risk of cancer (Araldi et al., 2015). The CBMN assay can simultaneously provide substantial information about different chromosomal damage parameters such as gene amplification, chromosomal breakage and rearrangement (Fenech, 2002b). It has been used successfully worldwide as an approved biomarker of *in vitro* and *in vivo* genome stability studies due to its sensitivity, reliability and low cost (Fenech, 2001). MN determination in peripheral lymphocytes is widely used in genotoxicology to assess chromosomal damage caused by various genotoxic agents (Fenech et al., 1999).

We therefore used the CBMN assay for the first time to determine the effects of MYR B (10μM) and MYR N (20μM) in lymphocytes particularly from pre-cancerous patients to confirm our results from the Comet assay.

As we used the two different particle sizes of myricetin (bulk and nano) and knowing that the toxicity of NPs is considered as a major concern when using them in genotoxicity studies, we therefore determined the cell viability and cytotoxicity of chemicals under test, using the trypan blue exclusion and MTT assay respectively, in lymphocytes from all the investigative groups including healthy individuals, pre-cancerous patients and multiple myeloma (MM) patients for 1, 24 and 48 hrs. Various concentrations of chemicals were considered. Analysis of results shows that cell viability did not drop below 80% in lymphocytes from healthy individuals and pre-cancerous patients at any considered concentration (chapter 3, figure 3.3). However, both forms of myricetin induced a time and dose dependent cytotoxicity in lymphocytes from MM patients when compared the healthy control only at higher concentrations. The cell survival decreased to 68% and 51% by MYR B (40μM) and MYR N (40μM) correspondingly after 48 hours of treatment (figure 6.1). Therefore, in the current study, only non-toxic concentrations with viability more than 80% (Henderson et al., 1998) were used to exclude any artefact effects due to toxicity. The genotoxic potential was also assessed employing the Comet assay where both forms of myricetin induced a concentration dependent genotoxicity only at higher concentrations. Indicated by the results only the optimal non-genotoxic concentrations determined by both parameters of the Comet assay (10μM for MYR B and 20μM for MYR N) were chosen to be used throughout the current *in vitro* study involving lymphocytes from healthy individuals, pre-cancerous blood patients and MM patients.

The results from the Comet assay have shown that the optimal doses used for MYR B (10μM) and MYR N (20μM) were non-genotoxic exhibiting no statistically significant DNA damage, in lymphocytes from pre-cancerous

patients, MM patients and those from healthy individuals when compared to their respective negative control groups(figure 3.5, 3.6, 6.2, 6.3). High levels of basal DNA damage were observed throughout the *in vitro* study in lymphocytes from patient group. When lymphocytes from pre-cancerous patients and healthy individuals were exposed to 50 μM H_2O_2, a known stress inducer, DNA damage was significantly increased which was substantially attenuated by MYR B and MYR N ($p<0.001$) addition (figure 4.4 A, B). However, MYR N has proven to be more effective against the damage. These results suggest genoprotective and anti-oxidant activities of MYR B and MYR N at lower non-genotoxic concentrations of 10μM and 20μM respectively, attributed by reduction in H_2O_2-induced DNA damage.

The effects of PhIP, in lymphocytes from pre-cancerous patients and healthy individuals were also investigated using the Comet assay. PhIP is an abundantly occurring HCA and a well-studied dietary mutagen/carcinogen and it has been shown to cause various types of DNA damage (Mimmler et al., 2016). PhIP treatment induced statistically significant DNA damage in lymphocytes from both groups ($p<0.001$)(figure 5.3 and 5.4). The patient group has shown increased sensitivity to PhIP may be due their compromised genome stability owing to the disease status (figure 5.4 a, b). MYR B (10μM) and MYR N (20μM) addition however, could significantly decrease the PhIP-mediated DNA insult and MYR N ($p<0.001$) has shown a better genoprotective effect against the damage. Results show the anti-mutagenic property of myricetin against PhIP-induced mutagenicity and that myricetin could protect the lymphocytes (both healthy individuals and pre-cancerous patients) from the deleterious effects of PhIP, ultimately protecting the cells from carcinogenesis, enhancing their repair capacity and promote the survival of the cells.

In the current study the results from the CBMN assay revealed that the frequencies of all the assay parameters such as MNi, nuclear buds and bridges were higher in pre-cancerous patient group at basal level compared to the healthy control group. Consistent with our results from the Comet assay MYR B (10μM) and MYR N (20μM) treatment induced no significant damage to the cells at chromosomal level; neither had they induced any nuclear buds or bridges (Table 3.6). Evaluation of the assay results shows that the incidence of MNi in BiNC from healthy volunteers and pre-cancerous patients declined when treated with myricetin bulk and the nanoparticle form but effect was not significant (Figure 3.8a). There were a few MNi seen in MoNC of healthy individuals which gives indication of little pre-existing damage due to lifestyle factors or any medical procedure undertaken. However, this number increased in the patient group pointing towards already present basal DNA damage. Myricetin nano (20μM) significantly reduced the MNi formation in MoNC from pre-cancerous patients when compared to the untreated group exhibiting its potential to reduce the basal damage in lymphocytes from pre-cancerous patients. Untreated healthy individuals have shown lower levels of DNA and chromosomal damage than the untreated patient group observed throughout the study in agreement with various past studies (Blasiak et al., 2004; Kontogianni et al., 2007; Stoyanova et al., 2010). The effects of PhIP on genome stability of healthy individuals' vs pre-cancerous patients was also investigated for the first time, to our knowledge, using the CBMN assay. Results demonstrated a significant increase in MNi formation in BiNC from both the investigated groups upon treatment with PhIP alone indicating the DNA damage was caused after the treatment (figure 5.3). Binucleated cellular division confirms the presence of a test chemical and DNA damage induced after

treatment (Doherty, 2012). However, MNi induction was inhibited to statistically significant levels when PhIP was combined with MYR B (10μM) and MYR N (20μM). MYR N was capable of causing better reduction in DNA damage, than MYR B. These results suggest that PhIP is a clastogenic, genotoxic and mutagenic agent which could potentially contribute towards the carcinogenesis in healthy individuals as well as in pre-cancerous patients. However, myricetin shows the ability to attenuate the harmful effects of PhIP by decreasing the DNA damage in a genoprotective and anti-mutagenic manner. Also, that MYR N (20μM) has shown better potential to bring these beneficial effects.

Since myricetin exhibits strong anti-oxidant activities in various cell systems and cell types (Semwal et al., 2016) we considered its anti-oxidant property as the mode of causing protection against DNA damage by supressing ROS related oxidative stress in lymphocytes especially from pre-cancerous patients. Oxidative stress in already known as a major cell signalling regulator of various pathways involved in cellular injuries (Schins and Knaapen, 2007) presenting it as a strong determinant of the effects of myricetin. Therefore, we investigated for the first time the effects of MYR B (10μM) and MYR N (20μM) on the intracellular ROS present at the basal levels, as well as after induction with the stress inducer, TBHP in lymphocytes from healthy individuals and those from pre-cancerous patients. Treatment groups (MYR B and MYR N) were compared against the untreated controls whereas the combination groups of MYR B or MYR N with TBHP were compared against the positive control (TBHP alone).

Evaluation of the results showed that myricetin in both forms significantly reduced the basal levels of ROS in lymphocytes from both the inspected groups (healthy and pre-cancerous), originally demonstrated in the current study (figure 4.5 and 4.6). Though, MYR N has shown better protection. TBHP induced the

intracellular ROS to a substantial significant level i.e. 40% in both studied groups. However, a significant attenuation of ROS was observed when co-treated with MYR B or MYR N. Again MYR N showed better capacity to protect the lymphocytes against TBHP-induced oxidative stress. These results were consistent with previous studies involving the protection of myricetin against TBHP-triggered cellular damage in various cell lines (Fraga et al., 1987; Pandey et al., 2009). Hence, the results suggest an overall anti-oxidant activity of MYR B (10μM) and MYR N (20μM) in lymphocytes from pre-cancerous patients and healthy individuals determined by a decrease in ROS levels upon treatment with myricetin particles alone and also against TBHP-induced effects.

Since GSH is the main anti-oxidant defence system in our cells, we assessed and analysed the effects of MYR B (10μM) and MYR N (20μM) on the basal levels of the enzyme in lymphocytes from both studied groups (healthy individuals, pre-cancerous patients) and on H_2O_2 or PhIP-induced alterations in the enzyme levels. *In-vitro* treatment of lymphocytes from healthy individuals and patients with stress inducers H_2O_2 (50μM) or PhIP (100μM) showed no significant effect on GSH levels or GSH/GSSG ratio. Co-supplementation of MYR B (10μM) and MYR N (20μM) also had no significant effect on the enzyme's levels in lymphocytes from either of the investigative group (figure 4.7 and 5.6).

The accommodation of approximately 2m long DNAs in roughly 10μM of nuclei of the human cells is made practical as a result of its organization into the chromatin by histone and non-histone proteins. This arrangement of DNA into compact chromatin is not only important for its functional effective activities but also for its regulation with other cellular processes (Kinner et al., 2008).

Alterations of chromatin have been widely studied with respect to transcriptional regulation but there is strong evidence now that chromatin structural modifications play a crucial role in the DNA repair mechanisms (Groth et al., 2007). Human cells are prone to endogenous and exogenous damage at all the times. DNA lesions or damage is categorised into two types depending on the degree of their effects on genome integrity. The first type includes, base or nucleotide damage as well as some single sugar backbone interruptions; an error-free repair could be easily accommodated to repair these lesions with the help of a complimentary DNA strand as a template with minor restructuring of chromatin and without much affecting the genome integrity. The other category, mainly includes DSBs, being lethal to cells are most deleterious types of DNA lesions which if left unrepaired could bring serious consequences for cellular survival and present a great threat to the genome integrity (Podhorecka et al., 2010). The induction of DSBs always triggers the initiation of several factors including the phosphorylation of the histone H2AX, a variant of H2A family proteins forming *γ*H2AX. H2AX is an element of the histone octamer in nucleosomes. H2AX protein is phosphorylated on the 139th serine residue in the presence of DNA damage (Rogakou et al., 1998) by ATM, ATR and DNA-PK kinases under varying circumstances in the PI3K pathway but ATM is the major protein that phosphorylate H2AX (Burma et al., 2001). Hence, *γ*H2AX is the initial step towards the recruitment of the DNA repair proteins upon induction of DSBs. Gamma-H2AX foci form in a 1 to 1 manner in response to DSBs formation and used as a biomarker of damage (Kuo and Yang, 2008). *γ*H2AX foci can be detected and visualised using a primary and secondary antibody immunocytochemically hence, gives the measure of DSBs formation. Evidence suggests that *γ*H2AX is a sensitive and initial biomarker of DSBs *in vitro* and *in*

vivo and it can detect low levels of DNA damage. The induction and repair of DSBs could be monitored. It could also be potentially used for monitoring and improving cancer therapies in future. Lymphocytes are the easiest and most commonly obtainable cells to detect *γ*H2AX foci *in vivo* ((Ivashkevich et al,. 2012). Under healthy conditions lymphocytes show very low levels of *γ*H2AX foci, typically less than one per cell. However this frequency increases upon induction of DNA damage in the form of DSBs (Geisel et al., 2008).

Therefore, we studied the effects of MYR B (10μM) and MYR N (20μM) on the basal levels of DSBs immunocytochemically using an antibody against *γ*H2AX, in lymphocytes from pre-cancerous patients compared to those from healthy individuals. We also investigated the gene expression levels of ATM kinase in lymphocytes from the pre-cancerous patients after *in vitro* treatment with both forms of myricetin using real-time PCR. Our results show that both forms of myricetin did not significantly induce DSBs in healthy individuals when compared to the untreated group (figure 4.8). Doxorubicin, used as the positive control has caused significant induction of DSBs in lymphocytes from both the groups' i.e. healthy individuals and pre-cancerous blood patients indicated by the number of *γ*H2AX foci formed per cell. Results showed an increased number of foci formation in untreated lymphocytes of pre-cancerous patients but the exposure to MYR B and MYR N could reduce their frequency but levels were not significant. Results suggest that myricetin does not induce DSBs in lymphocytes from healthy individuals and those from pre-cancerous patients at basal levels.

The results from real-time PCR show that there is no significant effect seen on the regulation of ATM kinase in lymphocytes from the pre-cancerous blood patients when treated with MYR B and MYR N (figure 3.9b). Research confirms

various crucial roles of ATM protein in cellular regulation including initiation of DDR, induction of apoptosis, triggering defence mechanisms against oxidative injuries and inhibition of ROS etc. (Semlitsch et al., 2011; Marinoglou, 2012). Studies also show that formation of DSBs leads to dimer dissociation and auto-phosphorylation of ATM kinase at serine 1981 (Rogakou et al., 1998; Bakkenist and Kastan, 2003; Helt et al., 2005). Research evidence also suggests that *γ*H2AX plays a significant part in recruitment of ATM to DSB location and consequent events leading to ATM-regulated cellular responses (Kobayashi et al., 2004). Based on our results from the Comet assay, micronucleus assay and immunocytochemistry we show that MYR B (10μM) and MYR N (20μM) do not induce DNA damage, MNi or DSBs in lymphocytes from both healthy individuals and pre-cancerous patients at basal levels.

ATR kinase plays an important role in phosphorylation of P53 and CHK1 in the presence of severe hypoxia and oxidative stressed induced DNA damage (Barzilai and Yamamoto, 2004). Therefore we investigated the effects of PhIP on ATR kinase regulation. PhIP has shown different effects on gene expression levels of ATR in lymphocytes from healthy individuals to those from pre-cancerous patients. The ATR gene was significantly ($p<0.001$) up-regulated by PhIP in healthy lymphocytes and it was further enhanced by myricetin supplementation where MYR N has shown $p<0.01$ significance (figure 5.7A). However, PhIP has demonstrated reverse effects on ATR expression in lymphocytes from the patient group. It significantly down-regulated the ATR gene levels ($p<0.01$) which were insignificantly up-regulated by myricetin addition (figure 5.7B). These results indicated that protective effects caused by myricetin on PhIP-induced damage might be dependent on the tumour-suppression activity of the P53 gene. Results also suggest that PhIP may cause

oxidative stress in lymphocytes from pre-cancerous patients that may be attenuated by ATR Kinase phosphorylation upon treatment with MYR B (10μM) and MYR N (20μM) dependent on P53. Thus enhancing the cellular capacity to recruit repair mechanisms before entering replication and maintain genome integrity.

But the mechanisms involved in diverse ATR regulation by PhIP in lymphocytes from healthy individuals compared to those from pre-cancerous patients are not fully understood.

The eukaryotic cell cycle is a highly organised and regulated process which allows cellular division and proliferation. It is mainly dependent on two decisive points also called checkpoints: one occurs in G1-phase where cells can undergo DNA synthesis after progression to S phase and the other comes at the G2/M boundary when cells are committed to DNA repair before entering mitosis (Huang et al., 2015). P53, a multifunctional tumour-suppressor protein is believed to play a crucial role in the regulation of transcription and the cell cycle (Darcy et al., 2008). The fate of the cells to experience cell cycle arrest at GI/arrest or apoptosis depends on the degree of DNA damage present in the cells. If damage is to a lesser content then the G1/arrest signal it is initiated by P53 to allow cells to undergo repair before committing to S-phase but if DNA shows dramatic damage then the death signal is triggered hence cells enter apoptosis. P53 is believed to trigger GI/arrest by transactivation and regulation of the expression of a cyclin dependent kinase inhibitor (CDKIs), p21 though controls the progression through the cell cycle (Haupt and Haupt, 2017). Although, P53 is well recognised as an effective transcription factor, it is also known to regulate some strong genes associated with the apoptotic pathway; the expression of Bcl-2, an anti-apoptotic protein can be repressed by P53 and

on the other hand, Bax being a promising candidate for its pro-apoptotic activities is characterised to be up-regulated by P53, triggering apoptosis (Kuo et al., 2006). The ability of P53 to differentially regulate specific proteins provides a complementary response to its transactivation activities.

Uncontrolled cellular proliferation in most of the tumour cells is due to their ability to evade apoptosis and cell cycle checkpoints. As P53 plays a crucial role in the healthy progression of the cell cycle, its mutation or deletion could lead to uncontrolled cellular proliferation giving rise to tumorigenesis putting tumour suppression at serious threat. The integrity of cellular DNA can also be compromised due to P53 inactivation hence leading to high mutational rates. P53 is a commonly mutated gene in most of the tumours resulting in various deleterious effects (Kong et al., 2012). Hence reintroduction of wt-P53 in tumour cells may present a promising strategy to overcome the resistance to apoptosis and control tumour cell proliferation. Increased levels of P53 may also help damaged cells to enter repair pathways before progression to mitosis thus repressing mutations (Shaw, 1996).

Therefore we further investigated the role of MYR B (10μM) and MYR N (20μM) on the expression levels of P53 protein at post-translational and mRNA levels in lymphocytes from pre-cancerous patients and MM patients compared to those from healthy individuals. We also investigated the effects on some of the P53 regulated genes; Bcl-2 and Bax while evaluating their regulation in lymphocytes from MM patients and healthy individuals.

Evaluation of the protein and gene expression was conducted using the Western blot analysis and the real-time PCR study respectively, in lymphocytes from patient groups' v/s healthy individuals. Results show that the P53 protein was significantly increased in healthy lymphocytes with MYR B (10μM) and

MYR N (20μM) treatment whereas its up-regulation in lymphocytes from pre-cancerous patients was less statistically significant at basal levels (figure 6.6 and 6.7). MYR B has shown better results than MYR N exhibiting 2.3-fold increase in P53 protein levels in healthy individuals when compared to the untreated group. However, in lymphocytes from pre-cancerous patients MYR N has been proven more effective than its larger particles, up-regulating protein levels to 1.4-fold (figure 3.10), consistent with the results from PCR where the P53 gene was significantly increased ($p<0.01$) in lymphocytes from pre-cancerous patients when exposed to MYR N treatment (figure 3.9a). *In vitro* treatment of lymphocytes from healthy individuals, with PhIP has shown significant down-regulation in the protein expression of P53 and Bcl-2. MYR B and MYR N addition, however, could attenuate this effect and increased both proteins to a significant level (figure 5.8). Consistent with our results from PCR where PhIP treatment significantly decreased the P53 gene expression, however, this was significantly up-regulated upon supplementation with MYR B and with MYR in healthy lymphocytes. In lymphocytes from the pre-cancerous patients the P53 gene was non-significantly down-regulated with PhIP treatment whereas significantly up-regulated upon MYR N addition ($p<0.001$) (figure 5.7).

Apoptosis is a route of programmed cell death (PCD) which is known to happen in multicellular organisms and plays a crucial role in maintenance of the normal cellular homeostasis. It presents an effective way to get rid of cancer cells. It has been demonstrated that reduced susceptibility of cancer cells to apoptosis is closely associated with poor prognosis of cancer (Hall et al., 2008). Hence, inducing apoptosis in cancer cells by means of natural compounds could be a promising strategy to overcome the increasing number of cancers worldwide.

Based on our results from the Comet assay we showed that both forms of myricetin, MYR B and MYR N at lower concentrations of 10μM and 20μM respectively, have not exhibited a significant genotoxic or genoprotective effects in lymphocytes from MM patients, when compared to the healthy controls. Thus, we further looked into the potential molecular processes mediated by myricetin in lymphocytes from MM patients. Myricetin has been shown in past studies to exhibit anticancer effects in various cancer cell lines possibly through its pro-oxidant activity (Pérez-Cano and Castell 2016; Semwal et al., 2016). We therefore, hypothesized that myricetin could potentially activate the P53-regulated apoptotic genes dependent on oxidative stress. Initiation of the intrinsic-apoptotic pathway mainly depends on the balance between the pro (e.g. Bax, Bad) and anti-apoptotic (e.g. bcl-2, bcl-xl) factors of the Bcl-2 family (Brunelle and Letai, 2009). Accumulating research has left no doubt that P53 plays various important roles in the regulation of different cellular processes such as cell cycle arrest, apoptosis, DNA repair and angiogenesis etc. (Dary et al., 2008). Therefore, increasing the amount of P53 may be a new strategy for treating cancer as it regulates the intrinsic apoptosis pathway such as Bax (kuo et al., 2006). Therefore, we analysed some of the factors involved in this pathway i.e. tumour-suppressor, P53, anti-apoptotic Bcl-2 and pro-apoptotic Bax in lymphocytes from MM patients comparing them with the effects in those from healthy individuals at basal levels. We investigated at both the mRNA and protein levels of all these genes.

In the current study myricetin bulk and nano forms were found to regulate the Bcl-2 family apoptosis-related proteins in lymphocytes from multiple myeloma patients but not in those from healthy individuals. Myricetin bulk and nanoparticles were observed to significantly increase the levels of P53 and Bax

while down-regulate the Bcl-2 expression in lymphocytes from myeloma patients at mRNA and protein levels (fig 6.5, 6.7). However, MYR B and MYR N regulate the expression levels of these genes differentially in lymphocytes from healthy individuals. P53 and Bcl-2 levels were significantly up-regulated while Bax was down-regulated at both protein and mRNA levels (Fig 6.4 & 6.6).

This might be due to differential effects of myricetin on healthy and patient lymphocytes. The lymphocytes from MM patient are possibly more sensitive to myricetin than the ones from healthy individuals due to their genome integrity or disease status. Our results are in agreement with a previous study where myricetin induced apoptosis in colon cancer cells by increasing the ratio between Bax and Bcl-2 protein levels (Kim et al., 2014).

In conclusion, both forms of myricetin (MYR B and MYR N) at lower non-genotoxic concentrations of 10 and 20μM respectively, have exhibited genoprotective, anti-oxidant and anti-cancer effects in lymphocytes from pre-cancerous blood patients, MM patients and healthy individuals. It showed strong anti-oxidant potential against H_2O_2 and TBHP-induced oxidative stress. It has also shown modulating effects against PhIP-induced genotoxicity and mutagenicity in lymphocytes from healthy individuals and pre-cancerous patients. Myricetin in both forms has exhibited anti-cancer and pro-oxidant potential in lymphocytes from MM patients by increasing the Bax/Bcl-2 ratio dependent on P53 and ROS, in the absence of cytotoxicity. In general, MYR N (20μM) has shown better protective effects than its larger particle size i.e. MYR B (10μM). This could be due to NPs seeming to be capable of passing through the biological membranes and inflowing the cell nucleus instead of the cytoplasm to a better extent compared to larger particles of the same material and afterwards NPs could gain access to the nucleus (Magdolenova et al., 2014) and becomes closer to the

DNA and consequently could be more effective (Chen et al., 2013). This could be checked in future using TEM analysis by studying the interaction of these particles with cells. The kinetics of their transport and communications with the cellular membranes are important factors affecting the function and biological effects of NPs (Manshian et al., 2017). The smaller size of NPs enhances their physio-chemical properties including, larger volume to surface area, reactivity in biological systems, solubility and stability etc. these properties could affect the activities of NPs by affecting their bioavailability in living systems (Franklin et al., 2007). The proficient effects of MYR N could also be possible due to its double concentration used than MYR B.

Collectively data from the current study suggests that myricetin supplementation in our regular diet with its enhanced bioavailability could have potential health beneficial effects and it could possibly protect and defend against various diseases including cancer. Effective formulation of myricetin as a food supplement or as a chemoprotective agent is possible after considering its physiological range, bioavailability, genotoxicity at higher concentrations and other biological factors involved.

7.2 Future work

As elaborated in chapter 3, myricetin bulk and nano forms have shown *in vitro* protective effects in peripheral lymphocytes from pre-cancerous patients compared to those from healthy individuals by exhibiting reduction in DNA damage and triggering the induction of P53 and ATM kinase. This requires further investigation of various other molecular processes and factors that may involve in protective effects of myricetin including P21.

There is *in vitro* evidence shown, of the strong anti-oxidant potential of myricetin bulk and nano forms in lymphocytes against H_2O_2 and TBHP-induced oxidative

stress as well as at basal levels. Therefore, further research on anti-oxidant defence mechanisms including the transcription factors, NF-kB and EpRE, and other intracellular anti-oxidant enzymes such as CAT would be useful to determine if myricetin also has an effect on the activity of these enzymes. Past study has shown positive effect of myricetin on regulation of these enzymes using various cell lines and in different disease conditions (Semwal, 2016).

The effect on DSBs formation has been shown in chapter 4, however, requires additional investigation of other repair mechanisms that may implicate the effects of myricetin in lymphocytes from pre-cancerous patients and healthy individuals. The Comet repair can be implicated by assessing the DNA damage at different time periods. Oxidative stress can also be investigated using FPG technique in future.

Myricetin has shown modulating effects against PhIP-triggered DNA damage by regulating P53, Bcl-2 and ATR kinase. This requires more research on the regulation of ATM pathway as a biomarker of DSBs formation. Research has shown that the ATM can be directly activated by DNA adducts and DSBs; hence, studying ATM will be usful to identify if PhIP induces DSBs in lymphocytes and whether myricetin has any protective effect against it (Choi et al., 2009; Kemp et al., 2011).

Evidence from chapter 6 showed that myricetin in both forms increase Bax/Bcl-2 ratio dependent on oxidative stress and P53 in lymphocytes from myeloma patients. Further study of the apoptotic cell numbers using TUNEL assay or flow cytometry systems would be beneficial to make a strong conclusion.

Chapter 8-References

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- (https://www.anthonynolan.org/patients-and-families/blood-cancers-and-blood-disorders/what-blood-cancer/lymphoma)

Appendix

Appendix # 1

Consent form

Title of Project: Molecular mechanisms of myricetin bulk and nano forms mediating genoprotective and genotoxic effects in lymphocytes from pre-cancerous and myeloma patients

Reviewed by Leeds Central Ethics Committee (REC) (REC reference number: 12/YH/0464)

1. I confirm that I have read and understand the information sheet for the above study. I have had opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by the individuals from the NHS trust or the University of Bradford, where it is relevant to my taking part in this research. I give permission to these individuals to have access to my records. ☐
4. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet. ☐
5. I agree to take part in the above study. ☐

Name of patient Date Signature
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Name of person
Taking consent Date Signature
---------- ---------- ----------

When completed, 1 for patient; 1 for researcher site file; 1 original to be kept in medical notes.

Appendix # 2

Participant information sheet for patients

Title of Project: Molecular mechanisms of myricetin bulk and nano forms mediating genoprotective and genotoxic effects in lymphocytes from pre-cancerous and myeloma patients

Reviewed by Leeds Central Ethics Committee (REC) (REC reference number: 12/YH/0464)

Invitation to the research study

We would like to invite you to take part in a research study. Before you decide to take part you need to understand the purpose of the study and how it would involve you. Please take time to read the following information carefully before considering.

What is the purpose of the study?

In this white blood cells (lymphocytes) will be isolated from the blood sample and treated with small chemicals particles to determine the potential effects of drug. A blood sample around 20mls will be taken. Samples will be stored for only the duration of study and used for studies with similar nature. The research is for PhD programme involving PhDs and post-doctoral fellows.

Why have I been invited?

You have been invited because you have disease states like and we would like to determine the possible effects of the chemicals used in your blood compared to the healthy individuals.

Do I have to take part?

It is entirely up to you to decide and sign the consent form to show that you agreed to take part. You can withdraw freely at any time without giving reason.

What will happen to me if I take part?

A small blood sample will be taken and you will need to attend the clinic again for this research study. Researchers will complete a brief questionnaire with you. We shall access your notes so that they can be linked in an anonymous way with your clinical notes. Each individual will be given a coded study

number. The data obtained will used by and kept with study team and will not be given back to you. Results will be compared on group basis. And data may be published in scientific papers. The work will benefit the medical and scientific community but will not benefit you as individuals. More information can be provided if required.

People who cannot take part in the study

People who are not well enough to take part will be excluded. If you have further questions you could contact the research team: Professor Diana Anderson, Established Chair in Biomedical Sciences, BSc, MSc, PhD, DipEd, FSB, FATS, FRC, Path, FIFST, FBTS, FRSM, FHEA, FRSC, University of Bradford, Richmond road, Bradford, BD7-2RR UK and Honorary Research Consultant to Bradford NHS Trust.

Dr Mojgan Najefzadeh MD, PhD post-doctoral fellow. Division of Medical Sciences, University of Bradford, Richmond road, Bradford, BD7-2RR UK.

Appendix # 3

Data collection form

Title of study: Molecular mechanisms of myricetin bulk and nano forms mediating genoprotective and genotoxic effects in lymphocytes from pre-cancerous and myeloma patients

Reviewed by Leeds Central Ethics Committee (REC) (REC reference number: 12/YH/0464)

SAMPLE NUMBER DATE OF SAMPLE

AGE

SEX (PLEASE TICK) M/**F** CONSENT INFORMATION SHEET

ETHNIC GROUP

OCCUPATION

CURRENT SMOKER Y/N PAST SMOKER Y/N

HOW MANY CIGARETTES PER DAY

CIGARETTES CIGARS PIPES

ALCOHOL Y/N UNITS/WEEK

VITAMINS/ANTIOXIDANTS

PRESCRIBED DRUGS

DIET WESTERN ASIAN OMNIVORE VEGAN VEGETARIAN

RECREATIONAL DRUG USE Y/N

IF YES PLEASE LIST

MEDICAL

CANCER/ INFLAMMATORY DISEASE

EXTENT SITE HISTOLOGY SURGERY

CANCER

INFLAMMATORY DISEASE

PRE CANCEROUS STATE

OTHER MEDICAL CONDITIONS

FAMILY HISTORY OF CANCER AND INFLAMMATORY DISEASE

CHEMOTHERAPY OR RADIOTHERAPY

MOST RECENT MEASURES

WEIGHT

HEIGHT

BMI

Appendix # 4

Equipment

Equipment and other materials	Company/Resources
Centrifuge Mistral 3000	MSE, Albertville, USA
CCD camera	Hitachi KPMI/EK Monochrome
Centrifuge (Biofuge 28 RS)	Heraeus, Sepatech, Germany
Coplin jars	VWR, Lutterworth, UK
Culture flasks	VWR, Lutterworth, UK
Cover slips	VWR, Lutterworth, UK
Electrophoresis tank (HU20)	Scie-Plas, Renfrewchire, UK
Dry incubator (37^0C) LKB BIOCHROM	Leec Ltd, Nottingham, UK
Freezer -20^0C	Sanyo, Ultra low, Japan
Freezer -80^0C	Sanyo, Ultra low, Japan
Falcon tubes	BD, Swindon, UK
Fluorescent microscope	Leica, Weztler, Germany
Fume hood ray air	Maiche Aire, Bolton, UK
Fume cupboard	Milton, UK
Ice maker(Scotsman AF 100)	Namur, Belgium
Komet 6 software	Kinetic Imaging, Nottingham, UK
Light microscope	Nikon, Japan
Incubator 37^0C with CO_2	Andor Technology Ltd, Belfast
Micro-centrifuge MSE	GMI, Alberville, USA
Microplate reader	Dynex Technology, Sussex, UK
Microscope (ortholux)	Leitz, Sturttgart, Germany
Pipettes	Gilson, Middleton, WI, USA
Mini protein gel electrophoresis	Bio-Rad, Hertfordshire, UK
pH meter	Dunmow, UK
Power pack supply	Pharmacia LKB, Uppsala, Sweden
Water bath	Grant Instruments, Cambridge, UK
Super frost slides	VWR, Lutterworth, UK
Zeta sizer-nano	Malvern instruments, UK
Microplate reader	Tecan, Switzerland

Appendix # 5

Abstract titles presented for scientific conferences

Shabana Akhtar, Rajendran C. Gopalan, Mojgan Najafzadeh, Mohammad Isreb and Diana Anderson. **Effect of myricetin nano and bulk forms on lymphocytes from blood cancer and pre-cancerous patients compared to those from healthy individuals**. Molecular Epidemiology Group, UK (MEGUK) Spring Meeting, Aberdeen, UK. March 2017 (Poster).

Shabana Akhtar, Rajendran C. Gopalan, Mojgan Najafzadeh, Mohammad Isreb and Diana Anderson. **Reactive oxygen species (ROS) induced oxidative damage in lymphocytes from healthy individuals and those from blood cancer patients: Protection by myricetin nano and bulk forms.** UKEMS Annual Meeting, Leuven, Belgium. June 2017 (Poster).